

13 March 1980

Dioxin and 2,4,5-T: what are the risks?

A court case due to take place on Monday will decide for, or against, the use of the herbicide 2,4,5-T in the United States. Two old adversaries, the Dow Chemical Company and the Environmental Protection Agency, will argue the case over the legality and validity of the partial ban on 2,4,5-T currently in operation in the US. Dow Chemical, the largest manufacturer of 2,4,5-T in the world, will challenge the ban imposed by the EPA in March of last year; it is Dow's view that 2,4,5-T poses no risk to users.

There are those in the EPA who now believe that Dow will win the case. The Agency's own study linking 2,4,5-T spraying with an increase in spontaneous abortions in the state of Oregon — information on which the decision to ban the herbicide was based — apparently has serious flaws. The study has been severely criticised on methodological grounds by many independent scientists, and at least three reports have been produced opposing the EPA's findings. They argue that the areas of land chosen in the original EPA report were not carefully matched and therefore could not be regarded as truly representative of a 2,4,5-T sprayed area and control area. In addition, differences in hospital admissions for miscarriages vary among the regions chosen for study, and these practices, critics charge, were not given due consideration in the original study.

Such criticisms may be justified, and a reappraisal of the Oregon study, taking these additional factors into account would probably remove the association between miscarriages and 2,4,5-T spraying.

Evidence that the EPA is indeed moving away from a reliance on the Oregon study is provided in the Federal Register of 13 December, 1979. The Agency now appears to be basing its decision to call for a total ban on 2,4,5-T on the fact that the 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) contaminant present in the herbicide is an established animal carcinogen. Many oncologists now consider that there is no safe threshold dose for a carcinogen, and the EPA considers, therefore, that to expose the public to such a chemical by permitting usage of 2,4,5-T poses an unacceptable risk.

There are many in the EPA who believe, however, that in spite of the presence of dioxin in 2,4,5-T there is still no hard evidence of a health risk from the herbicide. A recent request from Dow Chemical under the Freedom of Information Act for all of the EPA's documentation on 2,4,5-T could well provide confirmation of the lack of this evidence. If Dow's request is

granted, the EPA's case for seeking a permanent ban on the herbicide could well be undermined.

Evidence elsewhere against 2,4,5-T is not very strong either. The best study to date was completed in Sweden and produced evidence that among those exposed to 2,4,5-T and chlorinated phenols in the course of their work in the lumber industry, there was a six-fold increase in soft tissue sarcomas. But even in this study confounding factors may not have been taken fully into account and the Swedish government is reassessing it.

It is therefore quite clear that more good and reliable data need to be collected for a proper assessment of the hazards of exposure to 2,4,5-T and its contaminant, dioxin. The best place to seek such data is in the medical records of those who have been or will be involved in the manufacture of 2,4,5-T, since they are most likely to be at risk. In particular that is so because dioxin, which poses the greatest potential hazard, is generally present in larger concentrations during the manufacture of 2,4,5-T than during its use. And to conclude that 2,4,5-T is safe to use without reviewing the full evidence on industrial exposure, as did the UK Ministry of Agriculture, Fisheries and Food last March, is distinctly unsound.

At present the available industrial data are limited and concentrate on exposure after accidents. There is some evidence for a clustering of gastro-intestinal cancers among BASF workers who were exposed to dioxin in an accident in West Germany in 1953, and for an increased rate of heart attacks after an industrial accident in Amsterdam at the Philips Duphar plant in 1963. Monsanto have studied the after-effects of a 1949 accident, and concluded that among affected workers there was a normal incidence of cancer and a lower than normal incidence of heart attacks (*Nature* 14 February, page 613).

More industrial data are needed and must already be available both from the UK firm of Coalite and Chemical Products Ltd., whose reluctance to reveal its data we reported last week, and elsewhere. It is therefore essential that Coalite should reveal the study it has already done and conduct further studies using proper controls and including all those exposed to dioxin whilst in its employment. And when it has done so it must pass the data on to the two bodies which are accumulating a world data-base on 2,4,5-T: the US National Institute of Occupational Safety and Health and the International Agency for Research on Cancer. Only when good studies are complete and openly available can the air be cleared for or against 2,4,5-T. □



United States

Pressure mounts to resume chemical weapons production

REPORTS of the use of poison gas by Soviet troops in Afghanistan are being used to help generate support for allowing the US military to resume the production of chemical weapons banned since the late 1960s.

Supporters of such a move argue that there is considerable evidence that the Soviet Union already possesses a chemical warfare capability considerably superior to that of the US — and that the reports from Afghanistan indicates its willingness to use it.

Critics, however, argue that many widely-quoted estimates of the Soviet stockpile of chemical weapons are greatly exaggerated, and point out that there is as yet no confirmed evidence that Soviet troops have used anything more than riot control agents against Afghan forces.

The US army has been pushing for several years to proceed with production facilities for a new generation of so-called binary weapons, in which two non-lethal gases combine on firing or impact to form a deadly mixture. Such weapons have been under research since the mid-1960s.

Congress rejected this proposal in both 1974 and 1975. More recently, plans for \$111 million production facility was cut by the White House from the Defense Department proposed budget for the fiscal year 1981, currently before Congress.

But pressure is building for the proposal to be reinstated. And at the same time, the Pentagon is increasing the scope of the plans that it would like to put into effect. In January, Defense Secretary Harold Brown announced that, whereas previously it had been intended only to produce projectiles containing the two gases, plans are now being drawn up for producing binary bombs and warheads as well.

Last week the possibility of moving to production came even closer when William Perry, Defense Under Secretary for research and engineering, signed a memorandum instructing the department to complete designs for such a facility and draw up a construction schedule.

How sympathetic Congress is likely to be towards a request for funds for this purpose remains unclear. But a considerable number of articles have appeared in the US press in recent weeks focusing both on the claims about the use of poison gas in Afghanistan (which even the Central Intelligence Agency, after careful study, is said to have been unable to confirm), and more broadly on the apparent inferiority of the US's chemical weapons capability.

An article in *Time*, for example, states that "without a credible chemical counter-punch, it becomes more likely that the US

would have to resort to tactical nuclear weapons as a response to a Soviet attack".

Dr Matthew Meselson, professor of biology at Harvard University and a long-time opponent of chemical and biological warfare, claims that many of the current estimates — such as one that the USSR has a stockpile of 350,000 tons of chemical weapons, compared to only 42,000 tons in the US — are unrealistic and based on faulty speculation, even according to some Defense Department calculations.

"I am concerned that making policy by excitability is likely to lock us into a series of policies which are unwise from a defence point of view, since I do not think that

chemical weapons are that useful in combat situations", Dr Meselson said last week.

The pressure to reintroduce chemical weapons production has coincided with the latest round of chemical disarmament talks between the US and the USSR in Geneva. At the end of the last round in August 1979, US officials expressed optimism that real progress towards an agreement banning such weapons was beginning to be made.

Some now argue that, if the US steps up its production, this will provide added pressure for an agreement. Others, however, argue that it could create a new stumbling block to the negotiation. □

Princeton and Saudi universities sign life sciences agreement

PRINCETON University and the University of Riyadh in Saudi Arabia have signed an agreement under which each university will assist the other to build up a centre for training and research in the life sciences.

The Saudi Arabian university will donate \$5 million to Princeton, a substantial proportion of which will be used to modernise facilities in the university's Moffett Biological Laboratories, and the rest of which is likely to be used to support a number of new faculty posts in the biology department.

In return, Princeton has agreed to help the University of Riyadh to build up graduate and postdoctoral training and research facilities in the life sciences. This will be done both by advising on course curricula and the selection and training of personnel, and assisting with any necessary technical advice.

The agreement was signed in Riyadh last week by Princeton president Dr William G Bowen and the president of the University

of Riyadh, Dr Mansour Al Turki. It follows five years of negotiation between the two universities leading to what is believed to be the biggest single grant from an oil-producing state to an American university.

Dr Edward Cox, Chairman of Princeton's Biology Department, told *Nature* last week that the money would be used to provide additional support for fields of research, such as developmental biology, in which the university already has a strong research tradition.

Faculty members are currently debating the best way to use the new funds to support research salaries. "One view is that you could use the money for assistant professors, because it is the young people who are most in need of jobs", Dr Cox said. It might be possible to offer a number of five or six year fellowships.

In return for the financing, the biology department will assist the University of Riyadh to set up an international life sciences centre for postgraduate students and postdoctoral fellows, most of whom now have to travel abroad to receive their research training.

Several previous attempts to establish cooperative agreements between US universities and Middle East countries have stumbled over the refusal of the latter to accept non-discrimination clauses — leading to the fear that women, Jews and other minority groups might be excluded from participation.

The two universities have circumvented this problem by agreeing that scholars, technical experts and students will be proposed and received for exchanges "on the criterion of merit" rather than according to any other consideration.

David Dickson



"No!! We won't be needing any cheer leaders!"

Research agencies prepare to tighten budget belts

MAJOR cuts in the US science budget which could virtually eliminate any real growth in research funding over the next eighteen months are expected to result from President Carter's moves to reduce federal spending in an effort to lower the rate of inflation.

No cuts have yet been officially decided. But the President is expected to make public within the near future how he intends to move towards a balanced budget by eliminating about \$4 billion of federal expenditure in the current fiscal year by cutting back on commitments already made, and \$15 billion in the 1981 fiscal year which begins on 1 October by reducing the budget request to Congress.

Virtually the only area expected to emerge unscathed is the defence budget. And since the science budget is an area of discretionary expenditure — unlike social security, for example, where expenditure is mandated by law — it can expect to shoulder a fair part of the burden.

Research agency heads were asked last week by the Office of Management and Budget (OMB) how they would meet given targets for reducing expenditure. Among the cuts under discussion are thought to be:

- National Institutes of Health: OMB has asked for cuts of \$170 million in the rest of the 1980 budget, and \$340 million (about 10% of the total) in the 1981 budget as part of a \$700 million reduction for the Department of Health, Education and Welfare.

One immediate casualty could be the administration's attempt to maintain the number of new competitive research grants at about 5000. This may be reduced to about 3,500, considerably raising the necessary quality of applications likely to be funded. There would also be a general cutback across the whole range of NIH's research activities, possibly resulting in the loss of some scientists' jobs.

- National Aeronautics and Space Administration: OMB is said to have asked NASA to prepare for cuts of \$760 million, a sum equivalent to 14% of the civilian space budget. This would affect almost all of the NASA's programmes, in particular the launch of the space shuttle.

Various planetary missions, such as the Galileo launch to Jupiter, and the solar-polar mission to be carried out jointly with the European Space Agency, would have to be put back, and perhaps even eliminated. The gamma ray observatory, recommended as a new start for 1981, may also be affected.

- National Science Foundation: cuts in the order of \$90 million to \$100 million — about 9% of the proposed 1981 budget — are under discussion with OMB. The budget office has suggested that where possible basic research expenditures should be protected. The main impact is therefore likely to fall on areas such as applied

science and science education.

- Department of Energy: several large-scale energy research projects have to be abandoned or cut back. Among those being closely studied last week in solar energy, for example, were the "power tower" under construction at Barstow in California, and the Ocean Thermal Test Facility (OTEC), now nearing completion off Hawaii.

- National Bureau of Standards: OMB has asked for cuts of about \$7 million, and bureau officials have indicated that at the top of their list is likely to be a new \$5 million initiative to enhance basic research in the agency, a package of eight proposals included in the original 1981 budget request with the specific endorsement of

the Office of Science and Technology Policy.

Administration officials stressed last week that most of the potential cuts were still under negotiation. But President Carter warned state and city officials visiting the White House to expect some unpleasant surprises in the near future — and discussions were taking place last weekend to put together the final package.

If the cuts are made as expected, then they are unlikely to be strongly resisted by Congress, particularly in an election year when many voters will be looking for signs of financial stringency, and to oppose the cuts would be to oppose the President's anti-inflation strategy.

David Dickson

Environment

IMAGE
UNAVAILABLE FOR
COPYRIGHT
REASONS

Lowland tropical rainforest destroyed by road building in the Amazon Basin.

World Conservation Strategy sets priorities for global resources

THE latest international environmental initiative was launched simultaneously last week in 32 countries, amid hopes that the time is ripe to stop squandering the Earth's natural resources. In producing its *World Conservation Strategy**, the International Union for the Conservation of Nature (under the auspices of the United Nations Environment Programme and the World Wildlife Fund) has brought together a wide range of expertise to set out priorities for action to conserve and replenish global resources, from microbial cultures to tropical forests. The heaviest burden of such action would inevitably fall on developing nations, whose will may be impeccable, but whose means may be totally inadequate as the sponsors are bound to admit when they urge policy

makers to take up the strategy.

The major theme of the strategy — that conservation and development must go hand-in-hand has been gathering strength since the UN Conference on the Human Environment in June, 1972. Now the call is for governments and international agencies to base their development programmes on sustainable use of species and ecosystems, which means spending the interest while keeping the capital. The authors of the strategy cite familiar worldwide evidence that much of that capital is being dissipated. Many losses are due to overfishing in the oceans, overgrazing on the land, clearing of forests and destruction of wild animals and plants, often because people are too poor to replenish their resources, even if they take a long term view.

The way to reverse the trend is not by piece meal attempts to save resources once

*Available from the World Wildlife Fund, 29 Creville Street, London EC1, £3.60; also available as a book, *How to save the World*, Kogan Page, £2.95 paperback, £5.95 hardback.

they are threatened with destruction. According to the strategy, governments should consider the needs of conservation at all stages of development. As an example, the authors recommend that forestry should not be directed only towards increasing the yield of products and the provision of services such as recreation and education. Forest management must ensure that yields are sustainable, and genetic diversity is preserved. The preservation of genetic diversity in general is an immediate task for governments in order to prevent the extinction of further species of wildlife, crop plants and livestock, any of which could be needed in the future (for example, in agricultural breeding programmes or the production of drugs). The recommended approach is to preserve ecosystems intact within reserves, augmented by collections both of whole organisms, as in zoos and botanic gardens, and of genetic material such as seed and semen.

The preservation of genetic resources is also a priority for action by international agencies, which are called upon to provide a wide range of assistance such as that provided for crop plants by the International Board for Plant Genetic Resources. According to the strategy, financial assistance should also be sought from commercial enterprises that benefit from the exploitation of living resources.

Another international priority is action to halt the depletion of shared resources in oceans and river basins. The scale of losses also makes tropical forests and drylands a priority for international action. In the latter case, low rainfall, high rates of evaporation and inefficient use are encouraging the rapid spread of deserts. The strategy calls for the implementation of the wide ranging plan of action formulated at the UN Conference on Desertification in September, 1977.

The hazards that beset a scheme for environmental action, were well expressed by Mr Michael Heseltine, UK Secretary of State for the Environment, when he said at the launching ceremony in London:

"The basis of my approach — and indeed of the present government — rests on conservation, good husbandry and wise use of resources. But it would be less than frank of me to assert that all decisions of government flow naturally and easily from that premise".

It is, he pointed out, almost always a matter of balancing a developmental need, often with a clear economic advantage, against a potential conservation loss. Mr Heseltine injected a further note of caution when Mr David Attenborough, naturalist and broadcaster, insisted that the people at large should compel governments everywhere to recognise the strategy, which must become a political issue. Otherwise, Attenborough said, it was doomed to be "just another abandoned lofty plan". He has set his fellow citizens a difficult task.

Mary Lindley

United Kingdom

Fast reactor fuel cycle to begin

THE United Kingdom Atomic Energy Authority has been given approval to make regular shipments of plutonium nitrate in water solution from Dounreay on the north coast of Scotland to Windscale in Cumbria, north-west England, as part of an experimental fast reactor fuel cycle. The nitrate will be the result of reprocessing of fuel elements from the Dounreay prototype fast reactor (PFR). For economic reasons the Authority plans to refabricate new fuel, perhaps by the 'sol gel' process, at Windscale rather than at Dounreay. Shipments will begin later this year, at the rate of one every two to four weeks.

Behind the decision may lie uncertainty about the future siting of the commercial demonstration fast reactor (CDFR) that the Authority would like to build. The government has promised a public enquiry on the fast breeder before any commitment is made to the CDFR, and only if it were to be built at Dounreay would it be economic to build a fuel fabrication plant there.

The shipments ultimately will be on a large scale, involving some 800 kg of

plutonium a year. Each load, carried in a specially designed 250 litre flask in a 27m³ containment vessel, may amount to a critical mass of the metal — so the flask has had to be designed to minimise risk of criticality.

Another design problem has been the continuous radiolysis of water by alpha emission from the plutonium. This results in a build up of hydrogen and oxygen gas, in explosive proportions. An inert gas atmosphere is introduced to dilute the gas, and so reduce the risk of explosion.

"Nevertheless", says a Department of Energy note on the shipments, "assessments have been made of the possible consequences of gas ignition within the package".

The package is unlikely to be damaged in a transport accident sufficiently to breach the inner vessel, says the note. "However, if the vessel was ruptured, and in the further unlikely event that a source of ignition was present, and that the mixture of hydrogen and oxygen was in the explosive range, any resultant explosion internal or external to the containment vessel but inside the outer package would generate insufficient pressure to rupture the external structure."

The inner vessel is designed to withstand the pressure that, it is calculated, will build up in the vessel up to 250 days; then it is unlikely to rupture. The danger here is of a shipwreck, followed by a delay in recovery. The Authority has performed recovery tests on a dummy container hidden in a wreck off West Scotland, which "was found and retrieved in hours". However, tests have not been made on the time for which a damaged inner container (after a collision, say) would withstand pressure build-up.

In an impact test, however, there was cracking of the outer vessel and fracturing of the radiation shield. According to a report prepared by the Nuclear Installation Inspectorate, published last week "it can be argued that would give enhanced access to heat flux" in the case of an impact followed by fire. Although, said an Authority spokesman, "that was one we were a bit bothered about", the NII concluded that thermal tests that had been conducted (on an undamaged third-scale model) were "an adequate demonstration" of safety.

The greatest dangers may occur during the loading and unloading of the inner vessel to and from its impact-resistant container, where the Authority has assumed that the vessel would not survive a two-foot drop. But, says the Department of Energy, the Health and Safety Executive are satisfied, as a result of tests, that the vessel would survive such an impact.

Robert Walgate

Poland



Bartosiewicz: power cooperation

Energy agreement

MR Zbigniew Bartosiewicz, Polish Minister of Power and Atomic Energy has signed a cooperation agreement with Sir Francis Tombs, Chairman of The UK Electricity Council. The agreement covers the exchange of information and possible joint research on the more efficient use of conventional energy sources for power generation.

The Minister's five-day visit to the UK comes at a time when Poland's industrial expansion programme has outstripped generating capacity.

During his visit, Bartosiewicz attended a seminar organised by the Department of Industry, at which one of the major topics discussed was "down time" — the unscheduled stoppage of generating sets for maintenance work. UK participants in the seminar later reported that they felt the optimisation of maintenance logistics could well be a fruitful theme for cooperation.

Vera Rich

High-energy physics

Truce and a German director for CERN

PROFESSOR Herwig Schopper, presently director of the Deutsche Elektronen Synchrotron Laboratory (DESY) near Hamburg, has been designated director-general of the European sub-nuclear physics laboratory, CERN, Geneva. He will succeed the present joint directors-general, Leon Van Hove and John Adams, on 1 January 1981.

The appointment has not been without its drama. The Italian delegation, one of the 12 national representations on CERN's governing Council, had been refusing to accede to Schopper's appointment until possible conflict between future CERN and DESY programmes is resolved. But after an extraordinary statement from CERN and another from the Italian Ministry of Science, the air was cleared last week.

The CERN statement describes the meeting of the Committee of Council on 29 February. The programme for the construction of LEP, the large electron-positron collider which is CERN's next major project, was raised.

The statement said:

"The delegates at the Committee of Council agreed with the Italian delegation that LEP must have the top priority among the European accelerator projects in elementary particle physics, and that it should be built as soon as possible. The delegates also agreed, following an Italian proposal, that a study group be formed to discuss certain legal and budgetary problems and the relation of LEP to national programmes . . .

"The Committee of Council entrusted Professor Schopper with the mandate to present Council, at its session in June 1980, with his proposals concerning the top management structure and the directing staff of CERN . . ."

The 'legal and budgetary problems' refer to the question of whether LEP could be built within CERN's present budget, without declaring LEP as a project requiring special funding. The 'national programmes' referred to mean largely HERA, a proposal at DESY to build an electron-proton collider; its inclusion in the CERN text is something of a coup for Italy, which has been worried that HERA would compete with LEP.

In fact on many issues Germany and Italy have come extremely close, both wanting to push LEP along as fast as possible, at the lowest practical budget.

"I believe we can build the first version of LEP to give beam at the end of 1986", Schopper said on Monday. He will now participate actively in constructing the final proposal for LEP, which will be made to Council in June.

Whether LEP will be a CERN supplementary programme, in which the

participation of individual countries is optional, or part of the basic programme, in which it is not, is a crucial question, Schopper believes. "We must try to activate all sources inside CERN", he said, "and that requires flexibility to transfer people from one project to another". But if LEP is merely a supplementary programme, flexibility will be difficult to achieve.

Schopper thinks that LEP can be built "within a few per cent" of the current CERN budget of SF 590 million a year.

Italy reacted to CERN's announcement with its own statement. The Science Minister, Vito Scalia, said last week:

"... we are finally approaching the solution to defining the programmes of CERN. This has been the aim of the Italian government since November. . .

"For the first time in 25 years, CERN had found itself in difficulties in defining its programme against serious competition from other national laboratories. By the end of last year, the pressures we had been applying to reach a rapid clarification of these problems had had no appreciable result. Council opposed our proposal to

discuss the future of CERN, and the Italian delegation decided to underline its dissent. [The delegation walked out of the December Council meeting, an event unprecedented in CERN history. The meeting was to have appointed Schopper.]

"But last week the Committee of Council demonstrated unequivocally that our action, which was intended to give priority to the discussion of CERN programmes over and above the appointment of a new director-general, was fully justified . . .

"All delegates at the meeting of 29 February have given LEP an absolute priority so the new accelerator will probably be built in five years. This decision allows the laboratory to keep its position of pre-eminence and confirms the validity of the "active Europeanism" that the Italian government has done its best to promote in international research.

"In view of this development it is clear that the recent solution of the CERN case represents a success for my country . . . It clarifies also why the Italian delegation is now disposed to vote for Professor Schopper . . ."

Robert Walgate

Interferon

US company increases production ten-fold

THE US drug company G D Searle & Co announced last week that it plans to increase its fibroblast interferon production "six to tenfold" by the middle of 1981. Facilities are also being set aside for conjectured production by genetically engineered *Escherichia coli*. A new plant will be built at Searle's High Wycombe, UK, factory.

"But this project is purely for research and development", says Searle's director of research and biological development at High Wycombe, Dr Brian Richards. "It will cost us £6.75 million, and we don't intend to make a profit out of it".

Searle's increased fibroblast interferon production will be supplied to the Anderson Hospital of the University of Texas for clinical tests of the drug in cancer control. The trial has been designed to match an earlier one with leukocyte interferon; it is the first on cancer patients with interferon from fibroblasts, although there has been a successful trial on patients suffering from herpes keratitis of the eye (where the application was external). The difference between fibroblast and leukocyte interferon is not just that they come from different kinds of cells. By several laboratory criteria they also differ in structure. What is not yet clear is whether this will affect their therapeutic value.

Two-thirds of the space in the new plant

will be set aside on a conjecture: that within 18 months strains of genetically engineered *Escherichia coli* will exist which can compete with fibroblasts in interferon production.

"Charles Weissmann in Zurich has produced *E. coli* which manufacture one or two molecules of interferon per cell," says Dr Richards. "I'm quite satisfied he has the right gene product, but the problems are going to come in trying to improve the efficiency."

Searle has in fact constructed bacteria carrying other genes that are expressed with high efficiency — "we have cells producing haemagglutinin at 1% of total protein of the cell" says Richards. Enough is not yet known about expression efficiency to predict whether such bacteria containing the interferon gene in place of the haemagglutinin gene, would be equally efficient, but apparently it gives "cause for hope".

Searle's current interferon production is roughly one quarter of world production, including leukocyte interferon, he estimates. So with the increased fibroblast production alone, world interferon production will increase two or three-fold. "Ultimately costs might fall by a factor of 100 to 1000", Richards estimates. Interferon costs would then be on a par with those of antibiotics.

NEWS IN BRIEF

ESA chooses Hipparcos

THE Scientific Programme Committee of the European Space Agency decided last week to fund the astrometry mission Hipparcos as the next ESA mission after Exosat which is to be launched in 1981. Designed to improve the measurements of stellar positions by two orders of magnitude, the satellite will be launched by Ariane in mid-1986, and placed in a geostationary orbit for its lifetime of two and a half years. The total estimated cost of the project is 139.3 MAU (\$185 million).

Hipparcos was the Programme Committee's final choice in spite of a recommendation by the Scientific Advisory Committee to fund a dual mission consisting of experiments to measure the Earth's magnetospheric tail and a deep space flyby of Halley's comet. By a vote of 10 votes for and one abstention, the 11 member committee, consisting of delegates from each ESA country, decided to overturn the Scientific Advisory Committee's recommendation.

No evidence found of link between saccharin and cancer

No evidence of a positive link between the consumption of saccharin and an increased incidence of bladder cancer has been revealed by either of two studies whose results were published last week. One was carried out by Harvard University's School of Public Health and the other by the American Health Foundation.

The scientists who carried out the study, which involved surveys of the dietary habits of 592 and 367 bladder cancer patients respectively, point out that these results have two possible implications. Either saccharin does not cause cancer; or that its effects are too small to be identified by such studies.

However, writing in the *New England Journal of Medicine*, Dr Robert Hoover, director of a separate study carried out by the National Cancer Institute, states that "the evidence is that little, if any, current bladder cancer is due to the consumption of artificial sweeteners at the doses and in the manner in which sweeteners were commonly consumed in the past."

The Harvard study, reported in last week's issue of the journal, came to the conclusion that artificial sweeteners posed "little or no excess risk of cancer of the lower urinary tract", although pointing out that, since the latency period for cancer due to exposure to toxic chemicals can be 30 to 50 years, it will be some time before definite conclusions can be reached.

The Food and Drug Administration has proposed a ban on the use of saccharin following evidence that it can cause tumours when administered to animals at

high doses. However, the ban was held up by Congress in 1977 until further information about saccharin's effect became known.

Academy report attacked on solar energy predictions

A RECENT report published after four years of study by the Committee on Nuclear and Alternative Energy Systems of the National Academy of Sciences has been attacked for severely over-estimating the costs of developing solar energy as a major source of power — and hence over-stating the need to develop nuclear and coal technologies.

In its report, prepared for the Department of Energy at a cost of \$3 million and subject to some bitter internal disputes, the committee states its conclusion that solar energy "will probably not contribute much more than 5% to energy supply" in the US before the end of the century, at least not without massive government intervention to penalise the use of non-renewable fuels.

This conclusion, however, is being strongly contested by environmentalists, who last year persuaded the Carter administration to commit itself to policies for supporting solar energy research and development designed to meet 20% of the nation's energy needs by the year 2000.

In a letter to the *New York Times*, Gus Speth, chairman of the White House's Council on Environmental Policy, says that as far as he has been able to discover, the committee's estimate that the cost of reaching the administration's target was a prohibitive \$3,000 billion was the result of a "back-of-the-envelope calculation", based on assumptions such as all capital equipment being constructed at today's prices.

Reworking the calculation with declining prices predicted by the committee's Solar Resources Group reduced the cost by a factor of almost three, he said.

A spokesperson for the academy said last week that a reply to the CEQ's charges was being prepared and would be submitted to the *Times* for publication.

Canada appoints new science minister

JOHN ROBERTS, cabinet minister responsible for cultural affairs in Canada's last Liberal government, has been appointed Minister of State for Science and Technology in the new cabinet announced last week by Prime Minister Pierre Trudeau after the Liberal Party's success in the general election.

Roberts will also carry the portfolio of Minister of the Environment. This has disappointed officials in scientific and

professional societies who had hoped that the new government would, like its Conservative predecessor, appoint a minister with sole responsibility for science and technology.

However the Liberal Party has indicated that it intends to make the creation of a strong research and development effort in Canada one of its top economic priorities. An outspoken economic nationalist, Mr Herb Gray, who has been appointed Minister of Industry, Trade and Commerce, said last week that Canada's "disturbingly low level" of R&D expenditure was connected with the high level of foreign control of Canadian industry. Canada's Foreign Investment Review Agency, created in 1974 at Gray's suggestion, will have its mandate extended to investigate the R&D performance of such companies.

Livermore safety report criticised

OFFICIAL reports of "minimal damage" at the Livermore nuclear weapons laboratory from two earthquakes last January were criticised severely last week. Two structural engineers, John Rutherford and Gary Gray inspected the plutonium building where up to 300 lbs of plutonium are stored and described the damage as "severe". A wall had moved during the tremors (the largest of which measured 5.5 on the Richter scale) and it was in danger of collapse.

Friends of the Earth claimed that a big earthquake of the order of 6.5 on the Richter scale could cause a fire which would burn the plutonium causing it to disperse over a wide area in a radioactive cloud.

Dr John Gofman, of the University of California, and a former director of the laboratory, called the Livermore laboratory "a public health hazard". "There is a conceivable possibility of fire and the plutonium burning thus becoming airborne dust. To say, as the officials have, that nobody would be killed is nonsense", he said.

Sakharov vote avoided

THE Russian Academy of Sciences has avoided putting to the vote the expulsion of Dr Andrei Sakharov from its ranks. Two corresponding members of the Academy had proposed a vote but last week were persuaded to withdraw their proposal. Had a vote been forced, it would have taken place in secret and required a two thirds majority to succeed. There is only one precedent for the expulsion of a member of the Academy, and that was of someone whose original election was based much more on political grounds than on genuine scientific achievement.

FEATURES

Swedes vote on their nuclear future

On 23 March the people of Sweden will be voting in a referendum on nuclear power policy. **Wendy Barnaby** writes from Stockholm that the exercise is intended to meet party political rather than major policy objectives

AFTER more than seven years of increasingly acrimonious public debate about nuclear power, nearly all political parties are badly split on the issue and energy policy is totally stranded. Popular support for a referendum was building up at the end of 1978, encouraged by the anti-nuclear Centre Party and various environmental groups, but it was staunchly opposed by the other parties until the accident at Harrisburg. It then became obvious to the leader of the Social Democratic opposition, Olof Palme, that his only chance of winning the general election scheduled for September, 1979 was to about-face and support a referendum.

Faced with a parliamentary majority in favour of a referendum, the minority Liberal party government (which had already tabled an energy bill incorporating 12 nuclear reactors) had no choice but to go along with it as well. The Centre Party and the Communists, also anti-nuclear, argued that the referendum should be held at the same time as the election, but they were overruled by the combined forces of the Social Democrats, Liberals and Conservatives, all of whose leaderships are pro-nuclear, but whose backbenchers and rank and file are split. These parties would have been embarrassed to have their internal disagreements aired during the election campaign. So the referendum was scheduled for March, 1980 — possibly in the hope that the intervening northern winter and the spectre of oil rationing might have a salutary effect on the population's attitudes. (Sweden depends on imported oil for 70% of its total energy consumption, which amounts to a little more than 400TWh a year. Electrical energy, which makes up 90TWh of this, is produced mainly by hydropower. Nuclear power provides 25% of electrical energy; the rest comes from oil).

In the event, Olof Palme's about-face did not win him the election: the bourgeois bloc won with an overall majority of one seat. Nor were nuclear issues prominent in the campaign. They would, it was said, be discussed once and for all before the referendum. But it is clear that the referendum will not be the final curtain of the nuclear drama. It will simply set the stage for another long act.

After the formation of the new bourgeois government (a coalition of the Centre, Liberal and Conservative parties

with Centre leader Torbjörn Fälldin as Prime Minister), the referendum questions had to be drawn up. The party leaderships were divided essentially into two camps: the Communists and the Centre party opposing nuclear power, and the others in favour. But to draw up two alternatives would have meant that the Social Democrats backed the same alternative as the Conservatives. This was politically unpalatable to the Social Democrats, who feared they would lose voters if they were seen to be aligned with the Conservatives on such a crucial issue. They therefore wrote into their referendum manifesto that all power production should be state-owned: not a revolutionary demand, as about 85% of it is state-owned already. As expected, however, the Conservatives could not go along with this, so the pro-nuclear side split into two factions.

As a result of this political face-saving, the voters must now decide between three options. Option three, supported by the Centre and Communist parties, Christian organizations and a variety of environmental and radical fringe groups, advocates phasing out the six reactors presently in operation over a period of ten years, and intensifying energy-saving and investment in renewable energy sources. Option two, supported by the Social Democrats and Liberals, advocates "phasing out nuclear power at a pace possible, taking into account the need for electric power for the maintenance of employment and welfare." In practice, this pace would mean, according to option two, using the six reactors now in operation as well as the four ready for loading and the two under construction — a total of 12 — for their lifetimes (about 25 years), but not building any more.

Option one, supported by the Conservatives and industry, also advocates (in identical wording to option two), "phasing out nuclear power at a pace possible taking into account the need for electric power for the maintenance of employment and welfare". This pace would also mean using all twelve reactors. Officially option one also vetoes building another generation of reactors, but its backers make no secret of the fact that this is written in because it would be unwise to declare their enthusiasm for nuclear power in the present political climate. Option one is generally regarded as being more pro-nuclear and more likely to go ahead with



Two Swedish Prime Ministers: former Social Democrat Olof Palme (left) switched to supporting the referendum against his party line. New Centre premier Torbjörn Fälldin (right) is anti-nuclear, but could not implement an anti-nuclear decision

uranium mining in Sweden and breeder reactors than option two.

Having three options produces a situation fraught with difficulties. Although option three has been labelled the 'no' side, it is in fact advocating a further use of nuclear power — for 10 years. Options one and two have also labelled themselves 'phasing out' options, although they advocate a further use of nuclear power on a much larger scale than option three. The debate is not in fact about a 'yes' or a 'no' to nuclear power, but the number of reactor-years nuclear power should be allowed to stay. The public is not faced with a clear-cut choice. Another obvious difficulty with three options is how to interpret the results. The new government had a hard time appointing an Energy Minister, but they finally engaged the services of a non-party judge, Carl-Axel Petri. According to him, the support for options one and two will be counted together against option three. Option three must therefore poll more than the other two combined if it is to win. The leader of option three's campaign, Lennart Daléus, has said his option ought to be considered the winner if it polls more than either of the other two options singly. (The latest public opinion poll shows option one being supported by 25% of the population, option two by 34%, option three by 35%, with 6% undecided).

Although the referendum is advisory, all parties have said they will follow its result. But whatever the result, there will be political problems. It seems certain that options one and two will together poll more than option three. For Prime Minister Fälldin, opposition to nuclear power is a moral stance, and it is doubtful whether a referendum result could give him absolution to preside over a government taking another six reactors into operation. In the unlikely event of option three's winning, there would be other political problems. The Centre and Communist

parties do not have a parliamentary majority and would therefore be unable to enact the measures the people had voted for. This would probably lead, sooner or later, to a government crisis and new elections.

While the different factions squabble, the Swedish Council for Planning and Coordination of Research is trying to produce impartial, informative material. It has engaged pairs of authors with different opinions on energy questions to argue their cases on eleven topics, including how to store nuclear waste, how to heat houses, nuclear power and nuclear weapons, and sun, wind and biomass. Each of the pairs has a mediator whose task it is to clarify where the authors agree and disagree.

Whatever the outcome of the referendum, the campaign has demonstrated a lot about the treatment of a complex scientific and technical issue in a democracy. Rational argument about energy has been relatively unimportant. What has been important is politics.

Supporters of option three oppose nuclear power largely, it seems, because they see it as a symbol of many unattractive features of modern, post-industrial life: individual isolation, large-scale technology, powerlessness of the individual to determine living conditions, and so on.

Supporters of options one and two talk about the waste of investment if the country does not use reactors it has built. What they envisage is essentially a continuation of Sweden's post-war history: an economically-expanding society using the latest technology to secure personal welfare. □

THE Swedes estimate that the International Nuclear Fuel Cycle Evaluation (INFCE) has increased their chances of obtaining United States approval for reprocessing spent fuel from their reactors. Because they use American enrichment facilities, they are currently forbidden to reprocess spent fuel under the US Nuclear Non-Proliferation Act of 1978.

After the INFCE discussions, however, the US may well give Sweden permission — however guarded — to send spent fuel to existing reprocessing plants and leave the plutonium there.

All Swedish decisions on nuclear matters are in abeyance at the moment, pending the referendum. Irrespective of the result, however, nuclear policy will probably develop along the lines of rejecting a national facility for reprocessing and not insisting on having the plutonium processed abroad sent back. Sweden might well sell its plutonium to the countries which reprocessed it. Such a development would dovetail nicely with some sort of American permission to reprocess.

The two countries are negotiating a new agreement on nuclear cooperation, and it will be as part of this agreement that the reprocessing question will be raised.

Drug innovation— what's slowing it down?

The research and introduction of new drugs has been slowing down in the UK and the US. At the same time, government regulations on the safety of new drugs have increased. But **Fred Steward** and **George Wibberley** argue that there is not a simple relationship between these two trends

THE INFLUENCE of government safety and efficacy regulations on pharmaceutical innovation has become a prominent issue in relations between the industry and the British government. Last April the Association of the British Pharmaceutical Industry (ABPI), in its "election manifesto", criticised the government for producing "a cumbersome and expensive regulatory edifice, much of which serves little useful purpose so far as the well-being of the public is concerned". Vice-President Dr Peter Main singled out as a fundamental concern "the effect that the ever-increasing delay caused by regulations relating to the testing of new products was having on pharmaceutical research and innovation". The Association's news sheet has continued to give emphasis to the issue, accompanied by headlines such as 'Murder by Regulation' and 'Take off the Cuffs'.

Last September the new UK Health Secretary, Mr Patrick Jenkin expressed his concern that controls for reasons of safety, quality and efficacy "can reach a point where they are so thorough, so pervasive and so foolproof that everything comes to a full stop". He was anxious that "unnecessary impediments to innovation should be removed".

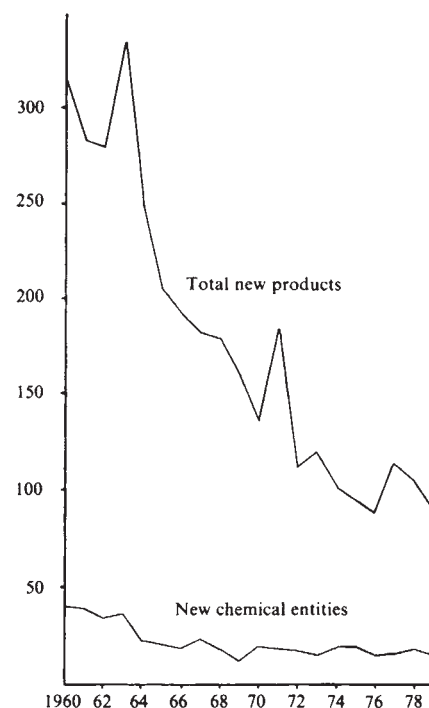
These views on the drug industry have been accompanied by mounting pressure against government legislation by other sections of British industry, and they have been anticipated in many ways by apparently similar arguments in the United States. The recent federal review of industrial innovation attributed negative effects to laws concerning health, safety and the environment, a conclusion much in tune with President Carter's commitment to "reduce, rationalise and streamline the regulatory burden". Attention has been focused throughout the 1970s on the impact of such controls on the performance of the drug industry, culminating in the introduction of a variety of proposed drug regulatory reforms in

Congress during 1978 and 1979.

The time is therefore appropriate to ask what exactly are the effects of regulation on innovation in the drug industry? Two points need to be made at the outset: much of the data and analyses have looked primarily at the US situation; and there are factors other than the legal environment influencing drug innovation. The Pharmaceutical Innovation Group at Aston University has been gathering data on drug innovation in the UK with the aim of evaluating the influence of regulation compared to other factors.

The annual rate of introduction of brand-name pharmaceutical products which doctors in general practice can prescribe in the UK has declined substantially from 1960 to the mid-1970s (Fig 1). This includes duplicates, new formulations, dosages and combinations of existing drugs. New chemical entities (NCEs) — products containing a new chemical substance — comprise only a small proportion of all new products. The annual rate of introduction of NCEs into

Figure 1: pharmaceutical products introduced into the UK 1960-79



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the UK market also declined in this period from about 30-40 to 15-20 per year; this rate remained reasonably steady throughout the 1970s.

Similar trends have been observed elsewhere. An analysis by Erika Reis-Arndt of the annual rate of introduction of NCEs into the world market showed a decline, mainly in the late 1960s, from nearly 100 in 1963 to 65 in 1973. The rate of introduction of NCEs into the US market, according to the data of Paul de Haen, shows an even sharper drop, from over 70 in 1960 to less than 20 in the 1970s.

The general decline in the rate of introduction of new drugs in the 1960s coincided with the introduction of stricter requirements in the UK and the US for the pre-market testing of new drugs. In the US the 1962 Kefauver-Harris amendments asked for evidence of efficacy in addition to evidence of safety already needed under the 1938 Food, Drug and Cosmetic Act. In the UK, a system for evaluating drug safety was introduced after the thalidomide disaster. The non-statutory Committee on the Safety of Drugs chaired by Derrick Dunlop began operating in 1964 and was replaced by a statutory licensing system involving the Committee on the Safety of Medicines under the 1968 Medicines Act. This put emphasis on efficacy as well as safety.

There are two particular difficulties in assessing the relationship between these regulatory changes and shifts in the rate of innovation. The first is that the large size of the US market and the importance of the US industry means that domestic regulations affecting them may have wider international repercussions; and the second is the fundamental problem of separating the influence of regulatory and non-regulatory factors on the pattern of innovation.

If there is a need for government intervention to prevent the marketing of inadequate drugs, then as a consequence of established control mechanisms, a decline in the introduction of inadequate products would be expected.

The greatest decline in new drugs in the UK has been of those containing an active ingredient already available in some other product. In the past, the medical profession has criticised the proliferation of similar and unnecessary combination products. The Committee on the Review of Medicines recently emphasised their lack of therapeutic value. There have been few claims that innovation in the formulation of existing drugs has been inhibited in recent years. Instead, the decline in the introduction of products based on existing drugs appears to fulfil the explicit objectives of regulatory policy.

The fall in the rate of introduction of NCEs is more difficult to analyse. By definition they possess some chemical novelty, but this is no guarantee that they all offer benefits in terms of safety or effectiveness. In the US, the Office of

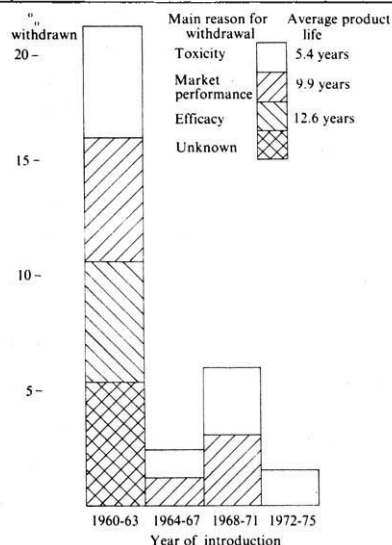


Figure 2: withdrawal of new chemical entities introduced in the UK 1960-75

Health Economics has obtained assessments by clinical experts of the therapeutic significance of NCEs introduced between 1958 and 1970.

Studying the introduction of NCEs in the UK, we found that the proportion in the bottom category of 'little or no advantage' dropped from 32% in 1960-63 to 18% in 1967-70. This included products which failed to meet the basic minimum standards set by the control system. Again, this decline appears to fulfil policy objectives. A classification by the Food and Drug Administration (FDA) of NCEs introduced in the US also showed a decline in the bottom category, representing 'no therapeutic gain', from 75% of 1961-1962 introductions to about 40% of introductions in the late 1960s and early 1970s.

It is remarkable that in the wide range of studies on regulation and innovation, relatively little effort has been devoted to exploring more thoroughly the degree to which the primary intended effects of regulatory change have been fulfilled. We have examined the rate of failure as shown by subsequent market withdrawal of NCEs introduced into the UK. The lack of attention given to this was illustrated early last year when the then Health Minister could not give any information in response to a parliamentary question about the extent of, or reasons for, the withdrawal of drugs from the UK market.

An NCE may be withdrawn for a number of reasons, including toxicity problems, inadequate efficacy and poor market performance. Our analysis of the withdrawal of NCEs introduced in the UK from 1960 to 1975 (Fig 2) shows a very much lower overall rate of withdrawal of NCEs introduced from 1964 onwards. Over 20% of the NCEs introduced in 1960-63, before the Committee on the Safety of Drugs started operation, were subsequently withdrawn from the market. Recent introductions may distort these

figures but the trend shown bears out the view that fewer drugs which ultimately failed reached the market after the establishment of stricter control.

The persistence of NCEs with unacceptable toxicity shows the importance of more effective surveillance of drugs when they enter medical practice. Some of the decline in the rate of introduction of NCEs in the UK therefore fulfils the goals of drug control policy.

The 1960s decline in the rate of NCE introduction, however, cannot be accounted for in this way. Increases in cost and length of time involved in discovering and marketing new drugs are also factors.

We measured the time from patent or publication, to the marketing of a new drug in the UK. Averaging about three years in the early 1960s, this rose to about 7.5 years in the early 1970s and remained then reasonably steady until a rise to nearly nine years in 1978-79. These increases have often been attributed to the demands of regulatory bodies, and it has been suggested that the overall decline in the rate of NCE introduction is a consequence of drug regulation.

But what are the other possible influences on the time and expense of drug research? A number can be suggested. The identification of many biologically important chemicals shortly after the war may have created a fruitful basis for innovation, for a period, but subsequently showed diminishing returns. Changes in the attention given to certain diseases could also have significant effects on the discovery and evaluation of new drugs. The complex aetiology of diseases such as cancer poses problems which cannot be resolved simply by expanding the screening of new compounds. The complexity of cardiovascular diseases again illustrates the difficulty of finding clear cut cures. There is, therefore, a need for more extensive evaluation of the effectiveness and toxicity of drugs intended for long-term administration.

Some of the areas in need of major innovation, such as inflammatory disease, raise the particular problem of suitable animal models for screening purposes. All these factors point to more complicated, time-consuming and expensive drug research. Another factor may be the rising expectation of researchers for more sophisticated and expensive equipment.

An analysis we have undertaken of the diseases for which new drugs are introduced in the UK shows a shift from infectious diseases in the mid-1960s to cardiovascular disease in the mid-1970s. Detailed information on pharmaceutical R&D in the UK is not readily available, but some figures have been published for the US by the Pharmaceutical Manufacturers Association. These show that in 1977 35.9% of total R&D expenditure was incurred by discovery-oriented work of synthesis, extraction, biological screening and pharmacological testing. Clinical

evaluation took 22.5%, toxicology and safety testing 9.2% and regulatory preparation and submission 3.3%.

Although the rate of introduction of NCEs stabilised during the 1970s, it is becoming harder to discover new molecules of real therapeutic importance. Of 83 NCEs approved by the FDA between 1974 and 1978, 62% were considered to offer moderate or important gains. This contrasts with only 33% of the 45 new molecules in pending new drug applications in March 1978.

Whatever the cause of increases in the cost and time of drug innovation, the consequence has been a decline in the "productivity" of R&D investment.

The influence of regulations in the US has received considerable attention, much of it concerned with different rates in the introduction of NCEs in other countries. William Wardell, in particular, has documented in detail a persistent "drug lag" in the US from 1963 onwards, shown by the high number of NCEs introduced in Britain but not marketed in the US, or only marketed there some years later.

Although the marketing strategy of a particular company determines the country in which the drug is introduced, many of the differences between the US and UK arise from differences in regulatory approach. There have been conflicts between the FDA and its critics over the therapeutic significance of the 'lag'. Some have argued that important new drugs such as sodium cromoglycate were excessively delayed while others have replied that the proliferation of very similar drugs, such as the non-steroidal anti-inflammatory drugs, has been avoided. Wardell has criticised the administration of the regulations and not the existence of the 1962 Amendments.

Perhaps an acknowledgement of all these points lies in the FDA's new fast-track approval process which allows important innovations to move more quickly through the regulatory system. The mean approval time for 'promising therapeutic advances' in the year beginning October 1978 was 19.4 months compared with an average of 39.2 months for those of 'little or no therapeutic gains'. But the speedier British system carries disadvantages. Of the four NCEs introduced in the UK since 1964 that were later withdrawn on the ground of unacceptable toxicity, three (ibunefac, practolol and alclofenac) were never introduced in the US.

Any comparison of the risks of adverse drug reactions with the risks of delay in the introduction of important new drugs, presents serious difficulties. The assumption that reduction in the risk of adverse effects from drugs must be accompanied by an increase in the time taken for their introduction is not necessarily valid. Labels for regulatory systems such as 'strict' or 'fast' should be used cautiously especially when comparing

one system with another.

The underlying weakness of many studies on the relationship between drug regulation and innovation is that they treat R&D as a "black box" and use highly aggregated measures of innovative output. Although correlations have been observed between factors such as regulatory change, development time, research costs, and the rate of innovation, they have not been accompanied by comprehensive explanations of what changes have been taking place within the research process.

The decline in R&D productivity has meant changes in strategy. The proportion of expenditure on "basic" as opposed to "applied" research in the US drug industry fell steadily over the decade 1968-78 from 15.4% to 11.4%. British data are not precise enough to detect such a trend. There also seems to have been a move away from less economically significant disease, such as those affecting small numbers of people, or those prevalent in poorer parts of the world. Our analysis of the introduction in the UK of drugs for uncommon illnesses as a proportion of all new drugs shows a drop from 7.1% in 1960 to 4.7% in 1971-76. These changes are taking place in the context of companies concentrating on fewer therapeutic areas. Jerome Schnee

has shown that those US drug firms which concentrated research on fewer areas produced an average of 1.3 NCEs in 1972-75 while those which diversified their research generated an average of only 0.67.

These changes in R&D strategy may have major social consequences. The shift from basic research may well result in just the opposite effect to the closer relationship between basic and applied science. Changes in the therapeutic categories receiving attention may mean a neglect of important breakthroughs in less commercially attractive areas. Although such research decisions have widespread repercussions, they are not subject to any direct form of social control. The proposition that less, rather than more, government intervention is required in relation to drug innovation is at least an arguable one.

The precise relationship between regulation and innovation has yet to be established, and it is unlikely to be determined by further studies which attempt to quantify the adverse effect of regulation but do not examine the full range of factors which may influence drug innovation. We are exploring research and innovation in the drug industry with this perspective in mind. □

Evaporation apparatus for concentrating drug solutions in use at the Wellcome Laboratory of Drug Metabolism, Beckenham, Kent, UK.



NEWS AND VIEWS

Neural tube defects: towards prevention and understanding

from Gill Morriss

ANENCEPHALY and spina bifida aperta are open neural tube defects (NTD) with an overall population frequency in Britain of 1 in 500 and 1 in 400 respectively, although there are marked geographical variations¹. Their aetiology is not well understood, so that prevention has, until now, been possible only through antenatal diagnosis (alpha-fetoprotein testing) and subsequent abortion.

In a recent clinical trial the expected incidence of NTD was greatly reduced after prospective mothers at high risk were given vitamin supplements before conception and during the early weeks of pregnancy. If these results hold up in more extensive tests, true prevention will become a possibility in many cases.

These results are also interesting scientifically as they indicate that environmental (in this case nutritional) factors may have a much greater weighting in an individual's predisposition to NTD than was formerly realised. It is therefore possible that relatedness to an affected individual increases predisposition to the same malformation through common environmental factors as well as through common genes. The interaction between environmental and genetic factors has also been highlighted in recent work on a mouse model for NTD.

Before discussing these reports in more detail, it is useful to consider the concept of predisposition to malformation as proposed by Carter¹. The combined environmental and genetic factors predisposing an individual to the risk of malformation during development ('individual' here refers to the embryo) are assumed to be normally distributed in the population for any specific congenital malformation. The threshold at which an individual's total liability will result in malformation, lies well away from the mean population level of predisposing factors. This distribution is indicated by the solid line in Fig. 1. There are subpopulations within the general population which have an increased risk. For example, an increased predisposition can be predicted for the relatives of an affected individual (the index patient). According to Carter's model, the risk will depend on the number of predisposing

genes they have in common with the patient: 50% for first degree relatives (siblings and offspring), 25% for second degree relatives (nephews and nieces) and 12.5% for third degree relatives (first cousins). Increased risk within a subpopulation is indicated in Fig. 1 as a shift of the distribution curve to the right, so that a larger number of individuals have above threshold levels of predisposing factors (broken line). (Fraser² has used a similar diagram to illustrate the shift in the normal distribution curve to the right when two teratogens interact additively: genetic/environmental interactions can also be depicted in this way.)

Evidence for the importance of genetic factors in the causation of NTD comes from family studies and from racial variations which are at least partially maintained after migration. Evidence for the importance of environmental factors comes from studies on the effects of social class, maternal age and birth order, seasonal variation and so on³. In reality, it is impossible to assess the relative importance of genetic and environmental factors, nor even to distinguish between them adequately. Families and close-knit communities tend to have many cultural factors in common and nutritional habits may be maintained after migration. The reasons for the marked geographical variation are similarly obscure. Even though the reasons for differences in predisposition are so poorly understood, the increased risk of recurrence within a family is well documented: for instance, in the South Wales study of Carter, David and Laurence⁴, 5.2% of siblings of an NTD index patient were themselves affected, compared with an incidence of 0.77% in the general population of that region. (The latter figure is itself high compared with other regions.)

It was on the basis of these studies that Smithells *et al.*⁵ were able to select a high-risk group of potential mothers to test the hypothesis that the social class gradient in incidence of NTD was due to nutritional factors. Their own earlier studies had indicated that red cell folic acid and leukocyte ascorbic acid (vitamin C) deficiencies in early pregnancy might be correlated with NTD⁶. 175 women who had previously given birth to an NTD infant, and who wished to become

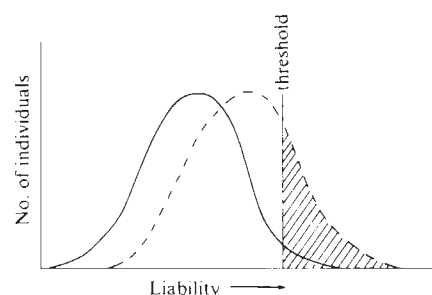


Fig. 1 Shaded area represents malformed individuals (see text). Modified from Carter¹.

pregnant again, were included in the study. They were given a multivitamin and iron preparation for at least 28 days before conception, continuing at least until the date of the second missed period, that is beyond the normal time of neural tube closure, which is completed 25 days after conception. (In view of the earlier suggested correlations with folic and ascorbic acid deficiencies, it is unfortunate that such a blanket preparation was used.) Only one of these mothers has produced a further affected infant. Fifteen mothers had an even higher risk than the rest of the group by virtue of having had two previous NTD infants; none of these had a further affected child. (The figures include 26 continuing pregnancies with normal amniotic fluid alpha-fetoprotein levels.) The recurrence rate of 1 in 178 infants (3 twin pairs) could be slightly higher than the 0.6% calculated, since there were 10 unexamined spontaneous abortions. Nevertheless the result is encouraging when compared with the recurrence rate in the control group (no vitamin supplementation), which was the expected number, 13 of 260 infants (5.0%).

Caution is essential at this stage: it is salutary to recall that the suggestion that anencephaly and spina bifida were 'usually preventable' by avoidance of blighted potatoes, was also supported by impressive statistics⁷. It is therefore very much to be hoped that the results of the nutritional supplementation study will be confirmed by extension of the trial to larger numbers of patients.

The concept of genetic and environmental factors affecting the teratogenic threshold has been highlighted recently in studies on the curly-tail mouse^{8,9}. This is a

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mutant with an incidence of spina bifida, exencephaly ($\equiv$ anencephaly) and related axial defects of around 50%. In outcrossing experiments with BALB/c and A strong random bred strains, the incidence of the curly-tail phenotype was reduced to 24% and 8% respectively (F_1 female backcrossed with curly-tail male parent), that is, the expression of the (recessive) gene was modified by the maternal genetic background.

The expression of exencephaly, unlike that of spina bifida, was strongly sex-linked, with an overall ratio of 0.25 male:female. A similar bias has been observed in another mouse mutant with a high incidence of exencephaly¹⁰, and the same has long been recognised in human anencephalics (ratio 0.34 in the South Wales study of Carter, David and Laurence⁴). These observations suggest that anencephaly involves a sex-linked gene-gene interaction, acting either on morphogenesis or on subsequent viability. Why this should be so for anencephaly and not for spina bifida is unclear, but it is apparent from morphological and ultrastructural observations that the sequence of mechanisms involved in morphogenesis of the cranial part of the neural tube is more complex than that of the spinal region¹¹. The striking morphological difference between the two regions is illustrated in a five-somite rat embryo in Fig. 2.

Excess vitamin A is well known as a teratogen which can affect neural tube closure when administered before or during the period of neurulation. Seller *et al.*⁹ have compared the effect of various dose levels of vitamin A given to curly-tail and A strong mice on day 8 of pregnancy. The effect of interaction of this environmental teratogen with the NTD-disposed curly-tail genotype was a shift of the teratogenic response to the right, that is, NTD resulted from lower dose levels in curly-tail compared with A strong mice. The higher dose levels, merely teratogenic in A strong, were lethal in curly-tail. This is a nice example of genetic/environmental interaction.

An intriguing result from another set of

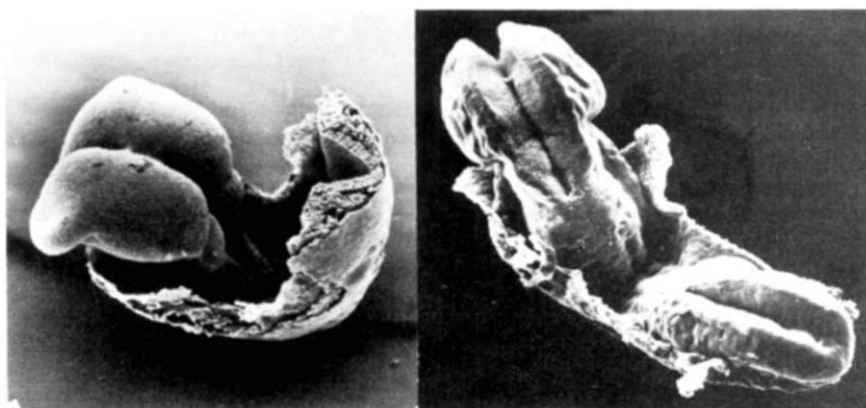


Fig. 2 Scanning electron micrographs of 5-somite (left) and 9-somite rat embryos. At 5 somites the cranial neural folds are relatively large, convex structures compared with the V-shaped neural groove of the spinal region. By the 9-somite stage, when neural tube closure has begun in the cervical region, the shape of the neural folds is similar in both cranial and spinal regions.

experiments in this series was the observation that administration of the two lower dose levels of vitamin A (5 mg kg^{-1} and 10 mg kg^{-1}) to curly-tail mice on day 9 resulted in a significant decrease in the proportion of malformed fetuses (33 and 36% respectively, compared with 54% in the sham-injected controls). This result is not to be compared with the effects of vitamin supplementation in high risk women — in the animal experiment we are considering a paradoxical genetic/environmental interaction, since administration of the same dose levels one day earlier had a potentiating effect on the genetic predisposition to NTD.

One must emphasise here that there are many possible interpretations of these observations, particularly since the neural tube of normal mice has completed closure by day 9, and it is usually assumed that open NTD are the result of failure of closure of the tube. Indeed, many previous studies on excess vitamin A have failed to induce defects following injection on day 9¹². It may be that NTD in the curly-tail mouse are associated with a propensity for delayed neurulation, and that vitamin A administered on day 9 interacts with the genetically-induced abnormal morphogenetic mechanisms in such a way as to increase the number of still open neural tubes which are able to complete closure at this late stage.

However, the possibility that there is secondary reopening of a closed tube cannot be altogether ruled out. At the region of closure (the dorsal midline) the closed neural tube and the surface ectoderm above it are both thin epithelia which are very easily broken during preparative procedures such as paraffin embedding, suggesting that a potential weakness may exist here in the living embryo. This said, it must be recognised that the weight of experimental evidence supports the assumption that open NTD usually result from failure of the neural tube to complete closure. This has been demonstrated in the case of excess vitamin A, for instance, by the addition of retinol

or retinoic acid to the culture medium in which neurulating rat embryos are developing¹³. Unfortunately vitamin A is of limited usefulness as a teratogen if one is searching for mechanisms at the cellular or molecular levels, because the molecular nature of its effects on morphogenesis is still obscure.

As one would expect in a system involving curvature of an epithelium, neural tube closure can be prevented by culturing embryos in the presence of substances such as cytochalasin B, which affect the contractile properties of actin-containing microfilaments¹⁴. But organised breakdown of microfilament bundles occurs in the most lateral cells of the neural epithelium in the last stage of curvature towards the midline before fusion during normal development; if this particular breakdown of microfilament bundles does not occur, neural tube closure again fails¹⁵. These contrasting observations illustrate the complexity of the process of neurulation, and make it clear that no single factor or group of factors can explain the cause and genesis of NTD.

Although the curly-tail mouse was first proposed as a model for human NTD twenty-one years ago¹⁶, no attempt has yet been made to investigate the morphogenetic basis of the observed abnormalities. The studies discussed above confirm the potential of this mutant, and demonstrate the interaction of genetic and environmental factors, but they do not provide any insight into the morphogenetic mechanisms underlying the NTD. Such insights will come only through painstaking observations of the embryos themselves, before and during the process of neurulation. Wilson and Finta¹⁷ have investigated the genesis of spina bifida in the splotch mouse mutant in this manner, and have found abnormally large numbers of gap junctional vesicles in the day 9 neural epithelium at the level which fails to form a closed tube. They discuss the possibility that these vesicles may be the visible result of an earlier process which has

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interfered with normal cellular contact mechanisms such as intercellular communication or adhesion; this interpretation is supported by the observation that the neural epithelium subsequently develops a looser structural organisation

than normal¹⁸.

Morphogenetic patterns and mechanisms in postimplantation embryos present serious problems of analysis because of their enormous complexity. However, we must come to terms with

these difficulties if we are to improve our ability to interpret and predict causes and origins of human malformations, and to be able to extrapolate from the results of experimental work on laboratory animals to man. □

Speckle interferometry of Pluto

from David W. Hughes

THIRTY years ago Kuiper used the 200-inch Mount Palomar telescope and a 'disk meter' to measure the diameter of Pluto, the ninth planet of the Solar System. He found a value of about 5,900 km but was considerably worried by possible systematic errors. The disk meter produces an artificial image of variable angular diameter, brightness and colour. These quantities are then adjusted until the artificial image and the image of Pluto look similar. The angular diameter of the planet can then simply be read off. Times change and in March 1978 S. J. Arnold and A. Boksenberg of University College, London and W. L. W. Sargent of the California Institute of Technology used the same telescope and observed the same planet but this time with a speckle interferometer at the Cassegrain focus. The value obtained for the diameter was $3,600 \pm 400$ km, this value however depending on the assumption that the planet disk was limb darkened. Their results have been published in a recent edition of *The Astrophysical Journal* (234, L159; 1979).

Speckle interferometry is a fascinating technique which has been developed extensively in the past 10 years and has been used to measure the diameters of stars, the separation of binaries and to study fine structure on the Sun and on the surfaces of supergiant stars. Atmospheric turbulence distorts the plane radiation wavefront from the astronomical object, a wavefront which has travelled more or less undisturbed through space. This corrugation serves to make a single large telescope mirror act as a multitude of smaller ones with individual sizes of around 10 cm. Small erratic movements are produced in the image position and the overall image is blurred, distorted and considerably enlarged. Turbulence is the main limitation to the resolving power of the telescope and the resulting image can be up to 80 times larger than the diffraction-limited image (the so-called Airy disk).

The speckle interferometric technique relies on taking many short exposure pictures of the object. This freezes the effects of turbulence such that the 'speckles' that make up each individual image are in essence distortion free. Each image is analysed statistically, the end result being a nearly diffraction-limited

image of the original object. Arnold, Boksenberg and Sargent took 170,000 'pictures' of Pluto, each having a 20 millisecond exposure. Each picture was produced by an image photon counting system consisting of a 256×256 element array which could find electronically the centre of each photon event. The information was recorded onto digital magnetic tape. Observations were centred on a wavelength of 5,000 Å with a bandwidth of 350 Å. There were about three photon events in each picture and each picture element was roughly equivalent to an angular extent of 4×10^{-3} arc s on the sky.

The image of Pluto was compared with two stellar images, one being Alpha Herculis A which had a known diameter and the other GC24617 which should be unresolved. The authors found a systematic error of 0.035 ± 0.010 arc s in their results which they thought might be due to an incomplete compensation for atmospheric dispersion and to the possibility that the speckles were not completely stationary over a period of 20 ms.

They conclude that the best fit to the profiles would be obtained if the disk of Pluto subtended an angle of 0.17 ± 0.02 arc s at the telescope and was limb darkened. The presence of limb darkening was deduced by Renschen (*Astronomische Nachrichten* 298, 179; 1977) from observations of Pluto's light variation as a function of time. The authors assume that this limb darkening follows a simple cosine law rather like that of the solar limb darkening. Titan, the 2,440 km radius satellite of Saturn, is also limb darkened like this. Arnold, Boksenberg and Sargent also found marginal evidence for an asymmetry in their data suggesting the presence of Pluto's moon. Also the surface of Pluto seems to have a variable albedo — half of it being covered with high albedo (0.5) methane frost and the remainder being of albedo about 0.13.

Combining the results with other recent observations leads to the conclusion that Pluto has mean opposition visual magnitude of 15.12, Pluto's moon being about 10.8^m. The mass is $(1.9 \pm 0.3) \times 10^{-3}$ Earth masses, the diameter is $3,600 \pm 400$ km and the mean density $0.5 (+0.3, -0.2)$ g cm⁻³. The low value for the mean density suggests that Pluto is made up predominantly of frozen volatiles. The

authors are obviously not too convinced about their limb darkening assumption and they carefully quote another set of results for the non limb darkened case. The mass obviously stays the same but the diameter and density change to 3,000 km and $0.8 (+0.6, -0.3)$ g cm⁻³ respectively.

As time progresses Pluto is looking less and less like a planet and more and more like an escaped planetary satellite. □

Soil seed banks

from Peter D. Moore

As any gardener knows, the soil seems to be full of seeds of a variety of species, some needing only warmth and water to germinate and others whose dormancy is not so easily broken. Harper (*Population Biology of Plants*, Academic Press, 1977) likens these respectively to a current and deposit account in a bank and the term 'seedbank' is one that is frequently used for this latent plant community.

A striking feature of the seed bank is the general disparity between the species contained in it and the present-day vegetation. Many examples could be quoted, such as the work of Kellman (*Can. J. Bot.* 48, 1383; 1970) who found that 70% of the seeds which germinated from soils beneath a century-old *Pseudotsuga* and *Tsuga* forest in British Columbia were *Alnus rubra*, many of the remainder being weeds. The possibility that the seeds had come in from other areas could not be excluded, but it was also considered conceivable that the seed population belonged to an early successional stage following a fire which had taken place a hundred years before.

Besides the disparity between present-day vegetation and seed bank, the seed bank itself can be extremely heterogeneous throughout the site. Van der Valk and Davis (*Can. J. Bot.* 54, 1832; 1976) used a Sorensen similarity index to compare the viable seed flora of mud samples from fresh water marshes in Iowa. Within marshes their indices varied from 2 to 68% and between marshes (eight were studied), indices fell between 24 and 28%. They account for the variability in terms of the time over which any given seed bank has accumulated and the local variation in

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vegetation history.

An exception to the vegetation/seed bank disparity has recently been described by Leck and Graveline (*Amer. J. Bot.* **66**, 1006; 1979) from the freshwater tidal marshes of the Delaware River estuary, New Jersey. These marshes contain a very diverse flora with annuals being particularly important; the tidal fluctuations render the habitat liable to regular disturbance by flooding. Annuals were common in the seed banks of these habitats, accounting for 7 out of the 10 most abundant species, and of the 10 most frequent species germinating, an average of 7.2 species were growing at the collection site.

This exception to the general rule may contain clues to the normal lack of correlation between vegetation and seed banks. The seed bank usually contains a high proportion of early successional (r-selected) species which rely on long-term viability as part of their opportunistic strategy. Late successional (K-selected) species gain no advantage from such persistence but must in consequence re-invade sites by new propagules following disturbance.

This is an oversimplification of what is evidently a complex of interactions, and a more extensive and systematic analysis has now been formulated by Thomson and Grime (*J. Ecol.* **67**, 893; 1979). They divide seed banks into four types (I-IV) which are thought to differ in their ecological significance. The types are defined by the temporal pattern of germination under normal field conditions. In species of type I and II the seed bank is non-persistent and temporary, seeds germinating either in the autumn after their production, or the following spring. Such types are associated with habitats in which regular gaps are formed seasonally and re-invasion depends on predictable, seasonal factors. In species of type III and IV there exists an increasingly large, persistent seed bank, which confers advantages under conditions where disturbance, and therefore the opportunity for germination, is unpredictable and not closely linked to season. It is natural therefore, that studies of the persistent soil seed banks show a predominance of weed species of essentially type IV. Where successional mature vegetation now dominates such sites, the contemporary dominants are unlikely to be well represented in the soil seed assemblage, for they are likely to possess type I or II features. This will result in the disparity so often observed between vegetation and the local seed flora. The exception, described from the New Jersey tidal marshes, experiences such frequent (but unpredictable) disturbance that the current vegetation is largely composed of type IV seed bank species as of course, is the soil seed bank. The model, developed on the basis of grassland studies, seems robust and will undoubtedly be applied with profit to many other habitats. □

DNA sequence of human papovavirus BK

from Peter M. Howley

THE papovaviruses, including Simian virus 40 (SV40) and its murine counterpart polyoma (Py), are small DNA viruses which have been well studied as models in tumour virology and to elucidate the mechanisms of eukaryotic gene expression. These viruses are oncogenic when injected into hamsters and can malignantly transform some mammalian cells *in vitro*. The first human papovaviruses were isolated in 1971; BK virus from the urine of a renal allograft recipient on immunosuppressive therapy (Gardner *et al. Lancet* **i**, 1253; 1971), and JC virus from the brain of a patient with a rare demyelinating disease, progressive multifocal leukoencephalopathy (Padgett *et al. Lancet* **i**, 1257; 1971). As BK and JC are also oncogenic in newborn hamsters and can transform certain mammalian cells *in vitro*, they have attracted interest because of their potential role in human neoplasia.

Serological studies indicate that BK and JC are ubiquitous in the human population; seroconversion occurs usually in childhood and 70–80% of adults from widespread geographical areas have antibodies specific for each of these viruses. Since BK can be readily propagated in a variety of cultures of human fetal cells and JC grows well only in cultures of primary human fetal spongiblasts (Padgett *et al. Infect. Immun.* **15**, 656; 1977) and to a limited degree in cultures of primary human amnion cells (Takemoto *et al. J. Virol.* **30**, 384; 1979), the molecular studies of the human papovaviruses have been largely limited to BK. Subsequent isolations of BK have been made exclusively from patients undergoing immunosuppressive therapy or suffering from an underlying immunodeficiency. (The laboratory strains of BK currently used are listed in Table 1.) The potential role of BK in human malignancy has been examined by several laboratories

and although conflicting reports have appeared, there is little convincing evidence to associate BK with human cancer (Fiori & DiMayorca *Proc. natn. Acad. Sci. U.S.A.* **73**, 4662; 1976; Israel *et al. Virology* **90**, 187; 1978; Wold *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 454; 1978).

The structure and genetic organisation of the BK genome are similar to those of SV40 and polyoma. The viral genome is a 3.4×10^6 molecular weight double-stranded circular molecule, equally divided into 'early' and 'late' sets of genes (for a recent review of the molecular biology of BK see Howley in *Viral Oncology* (ed. Klein) 489, Raven Press, New York, 1980). The early region, transcribed before the onset of DNA replication, encodes at least two polypeptides, large T antigen (97K) and small t antigen (17K) (Simmons & Martin *Proc. natn. Acad. Sci. U.S.A.* **75**, 1131; 1978). Three structural proteins (VP1, VP2, and VP3) are translated from the late transcripts.

Recently two laboratories have independently determined the complete nucleotide sequence of three different strains of BK: the prototype BK, BK(Dun), and BK(MM) (Seif *et al. Cell* **18**, 963; 1979; Yang & Wu *Science* **206**, 456; 1979). Comparison of these BK DNA sequences with the SV40 DNA sequence (Reddy *et al. Science* **200**, 494; 1978; Fiers *et al. Nature* **273**, 113; 1978) has provided definitive confirmation of earlier DNA-DNA hybridisation studies which indicated extensive sequence homology between the SV40 and BK genomes (Newell *et al. J. Virol.* **25**, 193; 1978; Howley *et al. J. biol. Chem.* **254**, 4876; 1979). The genetic organisation of BK seems identical to that of SV40 and a comparison of the BK viral sequences with analogous regions of the SV40 genome permits the localisation of coding regions and regulatory functions

Table 1 Strains of BK virus

Virus	Source	Reference
BK (prototype)	Urine, renal allograft recipient	Gardner <i>et al. Lancet</i> i , 1253; 1971.
BK(MM)	Urine and brain tumour, Wiskott-Aldrich syndrome	Takemoto <i>et al. J.N.C.I.</i> 53 , 1205; 1974.
BK(Dun)	Urine, Wiskott-Aldrich syndrome	Howley <i>et al. J. Virol.</i> 16 , 959; 1975. Manaker <i>et al. Virology</i> 97 , 112; 1979.
BK(RF)	Urine, renal allograft recipient	Dougherty & Stefano, <i>Proc. Soc. exp. Biol. Med.</i> 146 , 481; 1974.
BK(MG)	Urine, renal allograft recipient	Lecatsas & Prozesky, <i>Arch. Virol.</i> 47 , 393; 1975.
BK(GS)	Urine, renal allograft recipient	Wright <i>et al. J. Virol.</i> 17 , 762; 1976.
BK(DW)	Urine, renal allograft recipient	Wright <i>et al. J. Virol.</i> 17 , 762; 1976.
BK(JM)	Urine, Wiskott-Aldrich syndrome	Howley <i>et al. J. Virol.</i> 16 , 959; 1975.
BK(JL)	Urine, bone marrow allograft recipient	Pauw & Choufoer, <i>Arch. Virol.</i> 57 , 35; 1978.

for both lytic growth and transformation.

There are two regions where differences exist among the three strains of BK sequenced. The first is located in the non-coding tandem repetitive sequences to the late side of the origin of DNA replication. There is a 42 base pair deletion in the BK(Dun) genome compared with the prototype BK sequence. The BK(MM) genome varies more markedly from the other two DNAs in both the sizes and the patterns of these repeated sequences. Tandem repetitive segments are located in the corresponding region of the SV40 genome although they bear no sequence homology to the repeats of BK DNA. Variation also occurs in these tandem repetitive segments among different strains of SV40 and portions of these repetitive segments can be deleted without any apparent effect on the biological function of the virus. Thus there is apparently little sequence constraint in this region among papovaviruses generally or even among strains of the same virus, and its biological function and significance remain obscure.

Differences were also noted in the early region of the genomes of the BK strains. Although the sequences of the early regions of the prototype BK and BK(Dun) are identical, there is a deletion of 262 base pairs in the BK(MM) early region. This deletion removes sequences from the putative intervening sequence for large T antigen, including the likely donor site for the small t antigen splice junction. As a consequence, BK(MM) does not encode a small t antigen (Seif *et al.* *Cell* **18**, 963; 1979) making it equivalent to some of the constructed early viable deletion mutants of SV40 which map in the analogous region of the SV40 genome. Since BK(MM) can replicate in tissue culture and can induce tumors *in vivo* as well as transform cells *in vitro*, the small t antigen must be dispensable for these functions of BK.

While remarkable similarities can also be found between the genome of BK (or SV40) and that of polyoma, significant differences exist (Soeda *et al.* *Nature* **283**, 445; 1980). The BK and SV40 genomes contain a large non-coding region between the carboxy terminal ends of large T antigen and VP1 (approximately 100 base pairs), whereas the same region in polyoma contains only seven base pairs. In addition the polyoma genome does not contain the region of tandem repetitive segments in the non-coding region to the late side of the origin of DNA replication as do BK and SV40. The late leader regions of each of the BK and SV40 genomes contain an open reading frame following an initiator AUG referred to as the 'agnogene'. Although proteins encoded by these sequences have not yet been detected, approximately 2/3 of the putative amino acids are conserved

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Testing gravitational theories

from Bruno Bertotti

DURING their mission to Mars in 1976 and 77 the four Viking spacecraft verified Einstein's predictions concerning the additional delay in the propagation of an electromagnetic signal due to the gravitational field of the Sun with an accuracy of 2%, much better than any previous determination. A recent letter to the *Astrophysical Journal* (**234**, L219; 1979) by R.D. Reasenberg and others has now made public the details of this outstanding experimental achievement. This additional delay has the largest value, 250 μ s, when the signal grazes the Sun; with the Viking spacecraft (two orbiters and two landers) it was possible to measure corrections of 50 ns, corresponding to distances of 7.5 m. The main technical reason for this large precision was that the signals came from a lander, firmly anchored to the surface of the planet, whose motion is very regular and predictable.

This is another example of the extraordinary accuracy to which space research has pushed our knowledge of the geometry of the Solar System and, consequently, of such dynamical properties as the planetary masses; and there is still scope for improvement, mainly through the implementation of better telecommunication systems at higher frequencies (including the use of laser signals).

In my view this experiment marks also the end of one phase in gravitational research, which aimed at testing all the

theories of gravitation which are based on the assumption that space-time has a metric structure (described by its curvature) and that the Principle of Equivalence — all local laws have a universal validity — holds. These assumptions have been precisely formulated in a mathematical scheme, which has also led to the classification and the experimental consequences of all possible theories of this kind (see for example, Will, in *Experimental Gravitation* (Ed. Bertotti) Academic Press, New York, 1973). In recent years all these theories have been to a large extent falsified by experiment and theoretical arguments, except general relativity, whose predictions have all been confirmed to a greater and greater accuracy.

It seems, therefore, that the experimental programs within this theoretical framework have passed the point of diminishing returns. This does not mean, however, that in the physics of gravitation there are no open questions and potential anomalies. Perhaps the most important one is the possible change of the constant of gravitation G over a cosmological time scale, which has been extensively studied by P. Dirac and V. Canuto. If this is true, not only is Einstein's theory violated, but the whole of astrophysics and cosmology must undergo a drastic revolution. The tests of the constancy of G are not yet sufficiently precise to exclude this possibility; and precise geometrical measurements in the Solar System, especially if extended over a long period of time, will probably be crucial.

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between the BK and SV40 genome suggesting perhaps, that a protein exists. No corresponding 'agnogene' is present in the late leader region of polyoma.

The most significant difference between the DNAs of BK (or SV40) and Py lies in the genetic organisation of the early regions and possibly reflects the different host ranges of these viruses. The BK and SV40 early regions are extensively homologous throughout and each encodes only two known major early proteins, large T and small t antigens. The Py early region encodes a third early protein, middle T antigen (Ito *et al.* *Proc. natn. Acad. Sci. U.S.A.* **74**, 4666; 1977; Hunter *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 5917; 1978). Whereas the amino acid sequence of the Py small t antigen is remarkably similar to those of BK and SV40, the predicted Py large T antigen differs in two regions significantly from those of BK and SV40. First there is a 130 amino acid segment ('insertion') unique to the Py large T antigen located 100 nucleotides after the

early acceptor splice junction which has no counterpart in BK or SV40. The second difference is the carboxy terminus of the BK and SV40 large T antigens which is essentially absent from the Py large T. Interestingly the segment of DNA encoding this unique carboxy terminal portion in the BK and SV40 genomes is open in a second reading frame for approximately 300 nucleotides providing an additional potential coding segment. Putative peptides encoded by this segment of BK and SV40 would be hydrophobic and might thereby be used in associating a protein with a membrane; however, such peptides have not yet been demonstrated.

100 years ago

The following arrangements have been made at the Royal Institution for the lectures after Easter. Tuesdays: — Prof. Huxley — Two Lectures on Dogs, and the Problems connected with them; Mr. Robert H. Scott, F.R.S. — Four Lectures on Wind and Weather.

From *Nature* **21**, 11 March, 451; 1880.

More light on membrane protein mobility

from Michael T. Flanagan

ONE of the most useful and unambiguous techniques for measuring the mobility of proteins in a membrane is fluorescence recovery after photobleaching (FRAP). In this technique, a cell is labelled as specifically as possible by saturating a membrane-localised receptor with its ligand which has previously been labelled with a fluorescent dye. As described in the original papers, a small circular spot ($\sim 8 \mu\text{m}$ in diameter) is bleached on the surface of the cell by exposing it to a focused laser beam of the appropriate wavelength to be absorbed by and destroy the fluorescent dye. The appearance of fluorescence within the circle after bleaching can arise only from the diffusion of unbleached material into the spot. The rate of this recovery is related in a relatively simple manner to the diffusion constant of the labelled molecular complex (Edidin *et al.* *Science* **191**, 466; 1976; Schlessinger *et al.* *Proc. natn. Acad. Sci. U.S.A.* **73**, 2409; 1976). The essentials of the method were first described by Poo and Cone (*Nature* **247**, 438; 1974) who, in the absence of laser technology, measured the diffusion constant of rhodopsin in amphibian disk membranes using the easily bleached natural chromophore, retinal. They found a value approximately one order of magnitude less than that found, by resonance techniques, to characterise the lipids of natural membranes. The difference between protein and lipid mobility has emerged as the most interesting theme linking the various FRAP experiments.

A difference might be expected simply because the proteins are much larger than the lipids. However, the first FRAP experiments carried out on mitogens and antibodies bound to cell membrane receptors, for example, concanavalin A on myoblasts, IgE on mast cells, demonstrated that the mobilities of these protein receptor complexes were determined by more than their size. In general two populations of complex were found; one immobile and one with a diffusion constant up to two orders of magnitude lower than that of the membrane lipid. The diffusion constant of the latter population fell dramatically in the presence of cytochalasin B. This microbial metabolite does not alter the mobility of the membrane lipids. As it is known to combine with elements of the cytoskeleton these FRAP results strengthened the growing belief that there is a physical link between some membrane receptors and the cytoskeletal structure underlying the membrane. FRAP has also been used to study lipid mobility in its own right and such studies have given additional

confidence in the validity of the technique. The diffusion constants obtained using small fluorescent lipid probes confirmed the values obtained from magnetic resonance studies (Wu *et al.* *Biochemistry* **16**, 3939; 1977). This study also illustrated the limitations of the technique. Diffusion constants in liquid crystalline phases of pure lipids, for example, in dimyristoylphosphatidylcholine above 23°C , could be measured but not those in the gel phase, that lipid below 23°C . To extend the detection limit of the technique to cover these high viscosity membranes Smith and McConnell (*Proc. natn. Acad. Sci. U.S.A.* **75**, 2759; 1979) introduced a very elegant modification to the technique that has, in addition, assumed considerable importance in examining possible interactions between receptors and cytoskeleton.

Instead of bleaching a spot Smith and McConnell bleached a series of parallel lines by first passing the laser beam through a ruled grating. A square wave pattern is produced which rapidly forms a sine wave pattern. The diffusion constant is obtained from the decay constant of the amplitude of the sine wave. Not only is the sensitivity improved but gross cellular movements can be compensated for as the frequency of the discrete pattern is not altered by protein-receptor complex or labelled lipid diffusion. McConnell's group have now examined the mobility of the M13 phage coat protein incorporated into an artificial membrane at temperatures both above and below the phase transition temperature of the lipid. The results in the gel phase were compatible with either protein aggregation or phase separation into protein-rich and protein-poor domains. In the liquid-crystalline phase a diffusion constant for the protein comparable with that of the lipid was found (*Biochemistry* **18**, 2256; 1979).

In their next modification to the FRAP method McConnell's group returned to the problem of protein mobility in natural membranes. They have shown that it is possible to measure the anisotropy of diffusion in a membrane by bleaching a two dimensional periodic grid pattern (*Proc. natn. Acad. Sci. U.S.A.* **76**, 5641; 1979). The reduction of the data is no longer simple and requires Fourier image analysis. By aligning one axis of the bleached grid pattern with the predominant direction of the 'stress' fibres of mouse embryo fibroblasts showing anisotropic arrangements in their cytoskeletal components they were able to obtain the diffusion constants of the succinylated concanavalin A receptor complex in the directions both parallel and perpendicular to the 'stress' fibres. These differed by up to a factor of 10; the parallel motion being the faster. The presence of a physical link between this complex and the

cytoskeleton now seems certain. Whether it is a direct anchoring link or a more subtle one involving exclusion of the complex from certain regions of the membrane cannot be determined by this technique alone but FRAP clearly will be a powerful tool in the study of the fate of such complexes.

The factors that determine protein mobility in a complex membrane, even in the absence of cytoskeletal interactions, are not fully understood. This is in part because the structure and dynamics of the mixed lipid membranes themselves are not totally clear. But it is also impossible to predict with certainty how a protein will behave in a hypothetical continuous homogeneous two-dimensional fluid. The equations describing diffusion in three dimensions possess neat analytical solutions; when constrained to two dimensions they do not.

Saffman and Delbrück (*Proc. natn. Acad. Sci. U.S.A.* **72**, 3111; 1975) have examined the consequences of this paradox in the context of protein diffusion in a biological membrane. The availability of methods for the accurate determination of both lateral and rotational diffusion constants (correlation spectroscopy for the latter) should now allow the theoretical model derived by these authors to be tested and several of the groups using the FRAP technique see this as one of their goals. Experiments are also being devised to examine the effect of the multicomponent nature of the lipid membrane on protein diffusion. Vaz *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **76**, 5645; 1979) have taken the apolipoprotein, ApoC-III, as a model of a membrane protein and examined the effect of adding cholesterol to phosphatidylcholine bilayers on the mobility of the protein when bound to such bilayers. They believe that the protein is bound in the head group region of the membrane and that its mobility represents the upper bound for those values that will be found to characterise the integral membrane proteins.

Given the advanced state of the art of membrane reconstitution FRAP studies of several integral proteins are now feasible. In one of the first of these Schindler, Osborn and Koppel (*Nature* **283**, 346; 1980) measured the diffusion constants of the phospholipid, lipopolysaccharide and matrix protein in a reconstituted *Escherichia coli* outer membrane. They cannot reconcile their results with the model of Saffman and Delbrück and suggest that macromolecular diffusion in a membrane is more analogous to non-electrolyte diffusion through a polymer network than through a homogeneous fluid. FRAP experiments carried out on reconstituted systems containing enzymes whose activity has been shown to depend on the state or nature of the surrounding lipid, for example the ATP-dependent Ca^{2+} -transporting protein of the sarcoplasmic reticulum, will be of especial interest. □

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REVIEW ARTICLE

Highly excited atoms

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Highly excited atoms are oversize, fragile species currently studied for both intrinsic interest and as detectors of IR and microwave radiation. Traditional quantum mechanics provides accurate descriptions of atoms in these exotic states and modern dye lasers are responsible for the new capability in their spectroscopy.

DURING the past four or five years the spectroscopy of very highly excited states of atoms and molecules has advanced dramatically. These atoms are especially interesting because the active electron is much less perturbed by the other electrons in the atom than when it is in lower states, and the atom is much closer to the ideal hydrogen-like model so frequently used. Such highly excited atoms are very rare in nature because of their large size and fragility, but they are found in interstellar space (H I regions). The primary reason for the increase in the laboratory study of atoms in these exotic configurations is the recent availability of tuneable dye lasers which can provide beams of very high spectral density over the entire optical spectrum^{1,2}. These laser beams can be easily directed into high vacuum regions where atoms are relatively free of perturbing interactions, and can be tuned to excite the atoms to specific highly excited levels of interest.

Although series of atomic levels have been studied for nearly 100 years (Balmer described the regularity of the visible lines of hydrogen in 1884), most spectroscopy experiments have been restricted to the few lowest lying levels, particularly those accessible by electric dipole transitions from the ground state. Tuneable lasers allow direct excitation to high levels, permit stepwise excitation by two sequential electric dipole transitions to states not directly accessible from the ground state, and can induce second-order processes such as two quantum excitation to otherwise inaccessible levels. With proper choice of laser frequency, intensity, and suitable applied external fields, one may selectively populate almost any excited atomic state. Atoms in highly excited states are called Rydberg atoms.

In the following description of Rydberg atoms we shall use atomic units, (AU). Energy is measured in units of twice the Rydberg constant ($\approx 2.2 \times 10^{-5} \text{ cm}^{-1}$), charge and mass in units of the electron's charge and mass ($e = 1$ and $m = 1$), and time in units such that $\hbar$ also equals unity. Table 1 shows some common quantities measured in these convenient and ubiquitous units.

Table 1 Atomic units

$e = \hbar = m = 1 = \alpha c$
Unit of distance = $a_0 = \hbar^2/me^2 = 5.3 \times 10^{-9} \text{ cm}$
Unit of velocity = $\alpha c = e^2/\hbar = 2.2 \times 10^8 \text{ cm s}^{-1}$
Unit of time = $\hbar^3/me^4 = 2.4 \times 10^{-17} \text{ s}$
Unit of energy = $2R = mc^2\alpha^2 = 2.2 \times 10^5 \text{ cm}^{-1} \times hc$
Unit of E field = $e/a_0 = 5.1 \times 10^9 \text{ V cm}^{-1}$
Unit of B field = $c/\alpha = 2 \times 10^9 \text{ G}$

Atomic structure, Bohr model

Many properties of Rydberg atoms can be understood correctly in terms of the relatively simple notions used to describe hydrogen atoms. For example, the deBroglie hypothesis that an integral number of electron waves must fit on the circumference of a classical orbit gives the same formula for the energy levels derived many years earlier by Bohr, and this formula gives a very

good approximation to the energy levels of Rydberg atoms. We have $E_n = -(mc^2/2)(\alpha Z/n)^2$ which becomes

$$E_n = -1/2n^2 \text{ AU} \quad (1)$$

for $Z = 1$. The energies are negative and approach each other and zero at larger values of n , the principal quantum number. The energy separation between adjacent levels is approximately $\Delta E = 1/n^3$ and Fig. 1 shows the Bohr energy levels given by this formula. (Contrast these energies with the energies $E_n \approx E_0 n^2$ of a particle bound in a square well, where the energy levels spread further apart with increasing n and there are a finite number of bound states.)

In the Bohr model, the speed of the electron is $\alpha c/n$ which becomes $1/n \text{ AU}$, and the radius of the orbit is $(n\hbar)^2/me^2$ which becomes just $r = n^2 \text{ AU}$. The picture that emerges of a Rydberg atom is one of an ion core and an isolated electron very far away, floating lazily around in a slow orbit, much like a distant planet of the Solar System. For $n = 100$ the orbit is as large as a living cell and the electron speed is only $2 \times 10^4 \text{ m s}^{-1}$.

Although the Bohr energy formula gives very good values for the hydrogen atom, it does not do as well as for other atoms because the valence electron's orbit may take it inside the closed-shell core where the potential descends very much more steeply than that of hydrogen. The electron therefore has much lower energy as shown by the s-levels in the central part of Fig. 1. The low angular momentum states (penetrating orbits) of alkali metals are therefore much more tightly bound than either the high angular momentum states (non-penetrating orbits) which see a nearly Coulombic potential or the corresponding n states of hydrogen. The differences between the high angular momentum states of alkalis and those of hydrogen arise because the ion core is composed of mobile electrons whose orbits can be perturbed by the valence electron. In the language of classical electromagnetic theory, we say that the core is polarisable and that its polarisability influences the energy of the high angular momentum states³.

Although departure of alkali energy levels from those of hydrogen arises from two rather distinct mechanisms depending on the angular momentum of the alkali state, the energy levels E_n of all the alkali levels are given very accurately by a single, empirically determined, minor modification to the Bohr formula:

$$E_n = -\frac{1}{2(n-\delta_l)^2} \approx -\frac{1}{2(n^*)^2} \quad (2)$$

Here δ_l is the quantum defect for a particular angular momentum l and a particular atom, and is nearly independent of n . Some representative values are given in Table 2. We expand equation (2) for the case $n \gg \delta$ and find

$$E_n \approx -\frac{1}{2n^2} - \frac{\delta}{n^3} = -\frac{1}{2n^2} \left(1 + \frac{2\delta}{n}\right) \quad (3)$$

for the energies of the alkali energy levels which agrees very well with spectroscopic data.

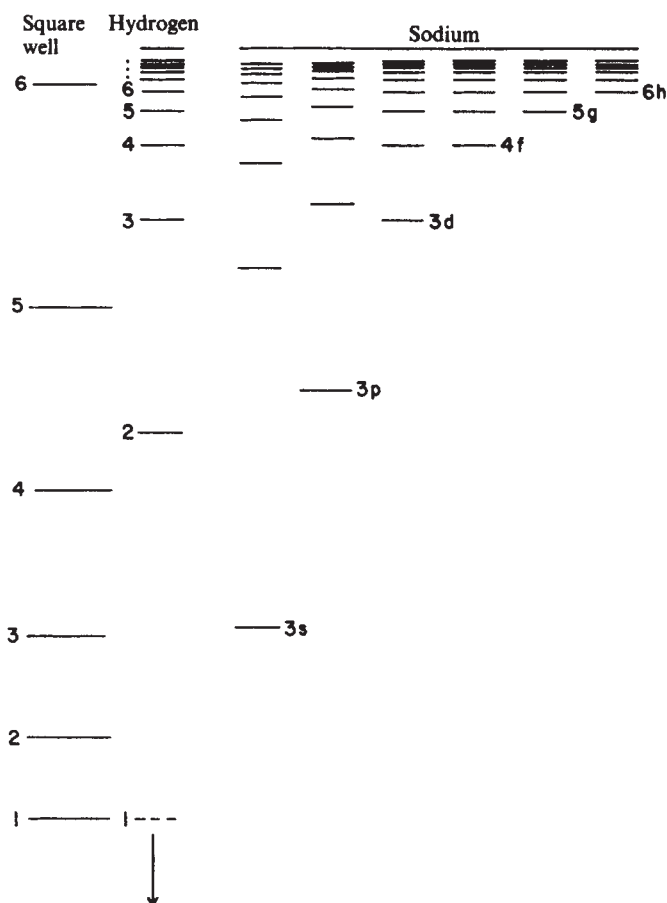


Fig. 1 Energy levels for various quantum mechanical systems. The left edge is a square well, the centre is the hydrogen atom, and the right half is the sodium atom.

Quantum mechanical discussion —no external fields

The quantum mechanical description of alkali atoms in Rydberg states begins with the Schrödinger equation for one electron in a Coulomb field, treating other effects such as spin orbit interaction (which decreases as $1/n^3$) as perturbations. The Schrödinger equation for hydrogen separates into a radial and angular part and the total wave function is the product $R_{nl}(r)Y_{lm}(\theta, \phi)$. The radial functions $R_{nl}(r)$ are the product of a Laguerre polynomial and a decreasing exponential in r . There are $(2l+1) Y_{lm}$ s that are solutions for each value of $l < n$ and that all have the same energy. Relativistic and radiative corrections produce the usual fine structure of these energy levels (spin-orbit splitting, Lamb shift, and so on) lifting the degeneracy among the states of different l and J . On the other hand, the effects of core polarisability and core penetration produces an electrostatic fine structure which also shifts the energy of states of different l , and these shifts are usually much larger than the relativistic ones. In fact, the spin-orbit splitting and Lamb shift are usually neglected in Rydberg spectroscopy because they are much smaller than other energy shifts (such as those arising from stray electric fields).

The quantum mechanical treatment^{3,4} of the perturbations on the valence electron in a high angular momentum state of an alkali atom begins with a derivation of the Hamiltonian term which arises from its interaction with the core. The electron polarises the core and the dipole thus induced acts back on the electron. An electron located at $r \gg r_{\text{core}}$ produces an electric field proportional to $1/r^2$ at the core, and the induced dipole moment produces an electric field at r proportional to $1/r^3$. Therefore, the additional field seen by the electron varies as $1/r^5$ and the potential of that field is proportional to $1/r^4$. We write

$$V_{\text{dipole}} = \frac{\beta}{2r^4} \quad (4)$$

where β (AU) is the classical polarisability and is of the order of unity (see Table 2).

We consider this potential as a perturbation to the Coulomb potential, evaluate its expectation values using the unperturbed wave functions of the hydrogen atom in the spirit of first-order perturbation theory (diagonal terms only), and find an energy shift

$$\begin{aligned} \Delta E_{\text{dipole}} &= \langle R_{nl}(r) | \frac{\beta}{2r^4} | R_{nl}(r) \rangle \\ &= \frac{\beta(3n^2 - l(l+1))}{4n^5(l+3/2)(l+1)(l+1/2)l(l-1/2)} \end{aligned} \quad (5)$$

For $l \sim n \gg 1$, $\Delta E_{\text{dipole}} \sim 1/n^8$ which is an extremely small correction to the energies. On the other hand, for fixed values of $l \ll n$ this term varies approximately as $1/n^3$, and the approximation is really very good. This is precisely the energy dependence needed to make the quantum defect formula equation (3) work, and shows why that formula can be very accurate for energies associated with induced dipole moments of the core. Of course, equation (5) is not valid for $l = 0$, but the energy shifts for s states (penetrating orbits) are dominated by the increased Coulomb energy and not by core polarisability.

The energy arising from the induced dipole moment is the largest contributor to the shift of alkali energies away from the hydrogen energies (for non-penetrating orbits), but there are other sources of energy shifts. For example, the electric field at the core from the valence electron is not uniform (it depends on $1/r^2$) and the response of the core to either a uniform or non-uniform field may not be linear. Therefore the induced charge distribution need not correspond to a pure dipole moment, but may have quadrupole, octopole, and higher order terms. For each of these there is an addition to the Hamiltonian which can be treated similarly to the dipole term. It is a very curious property of the hydrogen radial wave functions that, for all potentials, V , that vary as an inverse integer power of $r > 1$, the leading term for the energy shift is proportional to $1/n^3$ (ref. 5). The result is that the quantum defect formula of equation (3) provides a very accurate prediction for the energy levels from interactions of different types (penetration as well as polarisation) because multipole terms are all proportional to some inverse powers of r . Finally, neither the electron nor the core is at rest resulting in various dynamic contributions to the polarisability (see discussion in ref. 4).

Equations (3) and (5) can be used to find approximate values for the quantum defects of non-penetrating states:

$$\delta_l \approx 3\beta/4l^5 \quad (6)$$

and the $1/l^5$ dependence is consistent with spectroscopic data as exhibited by the entries in Table 2. We can therefore use the tables of spectral data to determine the dipole polarisabilities of the ion cores. The deviations from a perfect fit to equation (6) (or the unapproximated results of equation (5)) can be used to determine the quadrupole polarisability or other higher order effects (such as dynamic effects, and retardation). Furthermore,

Table 2 The core polarisabilities and quantum defects for several of the alkalis*

Core	polarisability (AU)	Quantum defect			
		s $l=0$	p 1	d 2	f 3
Lithium	0.19	0.4	0.05	0.002	—
Sodium	1.0	1.35	0.85	0.014	0.0015
Potassium	5.5	2.18	1.71	0.27	0.010
Rubidium	9.0	3.14	2.65	1.34	0.016
Caesium	14	4.06	3.58	2.47	0.033

*For more detailed information see ref. 9.

high resolution studies of Rydberg atoms with particularly simple cores (helium, lithium) can provide a basis for comparison between atomic theory and experiment.

Rydberg atoms in external fields

The description of Rydberg atoms in weak external fields is very similar to that of ground state atoms, but the field strengths that can be called weak are very restricted. As highly excited electrons are weakly bound, moderate external fields can have large effects on them.

Consider first a semi-classical description of the effects of a magnetic field. For very small fields the atoms exhibit the usual Zeeman effect. But larger fields induce considerable diamagnetism in Rydberg atoms as a result of their large size: their orbital magnetic moments $M = ia$ (a = area) scale as $(1/n)$ ($n^4 = n^3$ and the interaction is quite large).

A complete quantum mechanical discussion of diamagnetism in Rydberg atoms is beyond the scope of this review and some unsolved problems are now attracting considerable attention. An outline of some first steps will be presented here. Note that the Hamiltonian for an electron in a field is (in AU)

$$\mathcal{H} = \frac{1}{2}(\mathbf{p} - \alpha \mathbf{A})^2 + V \quad (7)$$

where $\mathbf{A}$ is the vector potential and V is the scalar potential of the electrostatic binding field. For $\mathbf{A} = 0$ the equation is separable; for a uniform magnetic field $\mathbf{B}$, $\mathbf{A} = \frac{1}{2}\mathbf{B} \times \mathbf{R}$. The $\mathbf{A} = 0$ solutions in spherical coordinates are also eigenfunctions of $\mathbf{A} \cdot \mathbf{P}$, the cross terms in the square in equation (7), and give the usual Zeeman effect. Although the A^2 term is negligibly small in ground state atoms, its r^2 (or n^4) dependence makes it significant for Rydberg atoms. When it cannot be neglected, the Hamiltonian is not separable in any coordinate system and a perturbation treatment is necessary.

We choose the basis set found from the solution of the separable Hamiltonian and evaluate the corrections to the energies from the Hamiltonian matrix elements. The first order diamagnetic shift⁶, found from equation (7), is

$$\Delta E_{\text{dm}}^{(1)} = \langle \psi | \mathcal{H}_{\text{dm}} | \psi \rangle = \langle \psi | (\alpha^2/8) B^2 r^2 \sin^2 \theta | \psi \rangle \quad (8)$$

where r and θ are the usual spherical coordinates and $\psi = R_{nl}(r) Y_{lm}(\theta, \phi)$ is the solution to the separated Schrödinger equation. To evaluate the angular part of this expression we use $\sin^2 \theta = (\text{constant}) Y_{2\pm 2} e^{\mp 2i\phi}$ and the properties of integrals of products of spherical harmonics. The radial part can be calculated from the Laguerre polynomials and the final result is

$$\Delta E_{\text{dm}}^{(1)} = \frac{\alpha^2 B^2}{8} \frac{n^2 [5n^2 + 1 - 3l(l+1)] (l^2 + l - 1 + m_l^2)}{(2l-1)(2l+3)} \text{ AU} \quad (9)$$

For $n \gg l \sim 1$, this becomes ($m_l = 0$)

$$\Delta E_{\text{dm}}^{(1)} \cong \frac{\alpha^2 B^2}{8} n^4 \text{ AU} = 5 \times 10^{-7} B^2 n^4 \text{ cm}^{-1} (B \text{ in Tesla}) \quad (10)$$

Observe that, for $n \approx 32$, the diamagnetic energy is comparable to the Zeeman energy (typically 1.4 MHz G^{-1}) at $B = 2 \times 10^{-5} \text{ AU} = 1 \text{ T}$. Also, the ratio of the diamagnetic shift to the Coulomb energy interval scales as n^7 so that these become comparable for $n \approx 42$ in the same field.

More accurate calculations of diamagnetic energy shifts require the off-diagonal matrix elements of the A^2 part of the Hamiltonian. It is clear from the $\sin^2 \theta$ dependence that the only non-vanishing angular integrals are those for which $\Delta l = \pm 2, 0$: A^2 can only connect states of the same parity and must satisfy a 'triangle condition' on the l values. It is also clear that $\Delta m = 0$ and that there is no selection rule on n . For strong fields and/or large values of n , perturbative calculations are not satisfactory and other methods of calculations are being explored.

Next we consider a semi-classical description of the effects of an electric field. Note that, even for very weak fields, there are

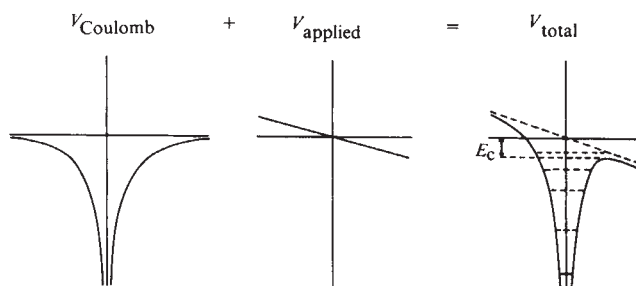


Fig. 2 The potential at the right results from adding the potential of a uniform field to that of the Coulomb field. E_c is indicated as the point where the electron is no longer bound to the force centre.

no perfectly bound states because there is always a finite distance from the atom where the electrical potential will be lower than the electron's binding energy. We therefore expect all expansions in powers of the field to diverge eventually. As an electric field acts on an atom through its induced dipole moment (which scales as r or n^2), and because the interaction is proportional to r , we expect energy shifts that scale as r^2 or n^4 and that can become quite significant in Rydberg atoms.

We begin a quantum mechanical description of Rydberg atoms in electric fields by noting that the Schrödinger equation can be separated in parabolic coordinates as well as the more common spherical coordinates even in the presence of a z -directed electric field⁷.

In contrast to the weak magnetic field case where the zero field eigenfunctions are also eigenfunctions of $\mathbf{p} \cdot \mathbf{A}$, the resulting radial differential equation for an electric field is not exactly soluble. Nevertheless, having the angular part separated makes the energy and eigenfunction calculations much easier. Parabolic coordinates are defined by

$$\begin{aligned} \zeta &= r + z = r(1 + \cos \theta) \\ \eta &= r - z = r(1 - \cos \theta) \\ \phi &= \phi \end{aligned} \quad (11)$$

We can write the solutions to the zero field Schrödinger equation as

$$u(\zeta, \eta, \phi) = u_1(\zeta) u_2(\eta) \Phi(\phi) \quad (12a)$$

where $\Phi(\phi) = e^{im\phi}$ as before and the energies of the levels are

$$E_n = -\frac{1}{2n^2} \text{ AU}, \quad n = n_1 + n_2 + |m| + 1 \quad (12b)$$

where n_1 and n_2 are the quantum numbers associated with the solutions for $u_1(\zeta)$ and $u_2(\eta)$.

In the presence of an electric field F the perturbation Hamiltonian is Fz (in AU) and we note that the matrix of Fz is diagonal in m because z commutes with L_z . In general the first-order perturbation correction to the energies is zero because Fz is an odd operator and has no diagonal matrix elements. However, hydrogen has some degeneracies between states of opposite parity which result in eigenfunctions of no definite parity, thereby producing finite diagonal elements. The first-order energy shift is then

$$\Delta E_s^{(1)} = \langle \psi | Fz | \psi \rangle = \frac{3}{2} \frac{Fn}{Z} (n_1 - n_2) \quad (13)$$

which scales as n^2 . In the more general case where there is no degeneracy, one must use the off-diagonal matrix elements and

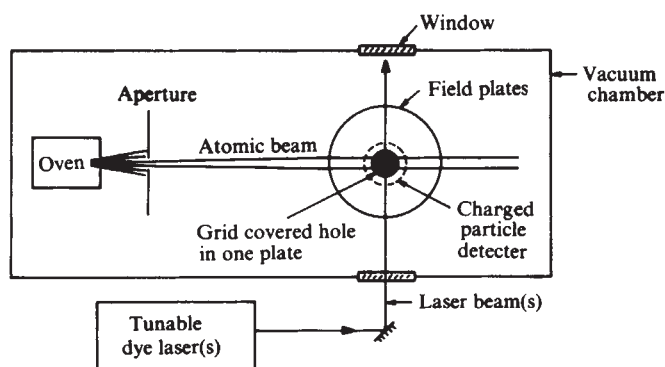


Fig. 3 A typical apparatus showing the major components.

second-order perturbation theory to find

$$\Delta E_s^{(2)} = -\frac{F^2}{16} \left(\frac{n}{Z}\right)^4 [17n^2 - 3(n_1 - n_2)^2 - 9m^2 + 19] \quad (14)$$

which scales as n^6 . The ratio of the second order Stark shift to the Coulomb energy intervals scales as n^9 and becomes unity for $F = 10^{-6} \text{ AU} = 5 \text{ kV cm}^{-1}$ at $n = 21$.

The Stark structure of the alkali Rydbergs is still being actively investigated. One can do higher order perturbation theory⁸, diagonalisation with a truncated basis set⁹, or other approximate calculations⁷.

Because of their relatively small binding energy, Rydberg atoms can be readily ionised by electric fields. We write the potential for a Rydberg electron as $V = -1/|r| - Ez$ (see Fig. 2) and find that it has a maximum value $V_{\max} = -2\sqrt{F}a$ at $z = 1/\sqrt{F}$. An electron with energy $-1/2n^2 > V_{\max}$ can escape. The condition for field ionisation is thus $F \geq 1/(16n^4)$ which occurs at $F = 10^{-6} \text{ AU} = 5 \text{ kV cm}^{-1}$ for $n = 15$.

Of course, field ionisation is not quite so simple. First, some electrons occupy states with wave functions concentrated away from the saddle-point produced in the potential by the electric field. They may have enough energy to field ionise, but do not sample the potential at the right place. Also, electrons with insufficient energy to ionise classically may escape by tunneling under the barrier. Further complications arise from the mixing of the wave functions by the field resulting in excited states that are superpositions of zero-field eigenfunctions. Field ionisation is a separate topic of serious investigation¹⁰.

Experimental description

The apparatus needed for the experimental study of Rydberg atoms is modest by comparison with other experimental facilities¹¹. One begins with a simple beam apparatus, consisting of a small stainless steel oven that can be heated to a few hundred degrees, contained in a vacuum system of characteristic linear dimension 0.5 m. Although some metals require a heat pipe oven, and some Rydberg spectroscopy has been done on rare gases which require no oven, the system described above is the most common. The atomic beam is collimated by a series of apertures and crossed by one or more laser beams which enter the vacuum system through appropriately placed windows (see Fig. 3).

At first it might seem that detection of Rydberg atoms could be very much the same as detection of other excited atoms: one simply looks at the radiation emitted when the atom decays to its ground state. More careful consideration reveals that Rydberg atoms do not decay very well by radiation, and in fact may have lifetimes long enough for the atoms to crash into the wall at the far end of the apparatus without ever emitting light. Transitions down to adjacent Rydberg levels are very weak and slow

because their frequencies are very low ($\Delta E = 1/n^3$) and spontaneous transition probabilities are proportional to the cube of the frequency ($1/n^9$). Transitions down to low lying or ground states, which have much higher frequencies, are very weak because the radial wave functions of Rydberg levels have very many oscillations and the integral of such oscillatory functions multiplied by one with only very few nodes is a small number, nearly zero. The result is that detection of Rydberg atoms by spontaneous emitted radiation is ineffective.

The usual procedure is to mount flat plate electrodes on either side of the atomic beam and apply a strong, d.c. electric field to the atoms. As the Rydberg electron is so far away from the nucleus and core, it is bound by a Coulomb field that is quite weak and it can easily be pulled away by the applied field and detected. Field ionisation, as described earlier, is very efficient for Rydberg atom detection.

Figure 3 shows a typical apparatus. The tuneable lasers are pumped by powerful beams from nitrogen or Nd:YAG lasers. The lasers are tuned to the transitions of interest and directed into the apparatus where they cross the atomic beam between the field plates. Time delay circuits triggered by the laser pulse apply the ionising field to the Rydberg atoms sometime after they have been created, and the detector measures the number of ions or electrons produced.

Applications of Rydberg spectroscopy

The signals are typically studied as a function of laser frequency, applied d.c. electric⁹ or magnetic fields^{15,16}, applied r.f. or microwave fields¹²⁻¹⁴ (which induce transitions between fine structure levels or to other Rydberg levels), background gas pressure¹⁷, or combinations of these and other influences. Figure 4 shows signals taken as a function of laser frequency at different values of applied electric fields (much smaller than the ionising field)¹⁸. Each vertical line is a wavelength scan taken at a different value of the applied electric field. The Stark spectrum of the sodium atom is dramatically laid out in Fig. 4.

The new knowledge which has come and will continue to develop from the study of Rydberg atoms spans many areas of atomic physics. For example, Stark shifts are greatly enhanced in highly excited states because the electron wave function is spread out over such a large region. The n^6 dependence described by equation (14) predicts a quadratic Stark shift of about $2 \times 10^{-3} \text{ AU} = 405 \text{ cm}^{-1}$ for an $n = 35$ atom in a field of $10^{-6} \text{ AU} = 5 \text{ kV cm}^{-1}$. As modern pulsed dye lasers can easily resolve 0.1 cm^{-1} (continuous wave (c.w.) dye lasers can resolve $3 \times 10^{-5} \text{ cm}^{-1} = 1 \text{ MHz}$), the Stark shifts arising from fields as small as 80 V cm^{-1} are quite noticeable in pulsed laser spectra, and from stray fields as small as 1.5 V cm^{-1} in c.w. (or r.f.) spectra. The Stark spectra of Rydberg states of several alkalis have been recently studied and described in detail¹⁸.

The Zeeman effect has no such spectacular enhancement. The magnetic shift of atomic energy levels is proportional to the Lande g -factor which depends only on the spin and the angular part of the wave function $Y_{lm}(\theta, \phi)$, and varies by no more than a factor of three over almost all atomic states. However, we have seen that the diamagnetic term or quadratic Zeeman term becomes important because of the large area (proportional to n^4) of the electron orbit. Diamagnetic effects of atoms in low lying states can only be observed in extraordinarily large magnetic fields, but for Rydberg atoms the effects are easily measured in laboratory fields^{15,16}.

When atoms are subjected to extremely strong electric or magnetic fields, that is, those fields which produce forces comparable to the Coulomb binding force, their structure is markedly changed. When the external field is so large that it can no longer be considered a perturbation, the ordinary solutions to the Schrödinger equation are not even approximately right. Theoretical studies of atoms in such conditions have been

stimulated by interest in plasma and astrophysical problems, but experimental measurements have not been done because laboratory fields can not be made large enough¹⁹. Rydberg atoms can provide a testing ground for the theories of atoms in strong fields because laboratory fields can easily be made to satisfy the strong field condition. We have already seen that field ionisation, that is, the process of atoms being torn apart by an external field, is a routine phenomenon in Rydberg studies. Further experiments should provide insights into the behaviour of matter at the surface of neutron stars (magnetic fields $\approx 10^{12}$ G) and in hot plasmas (very strong electric fields).

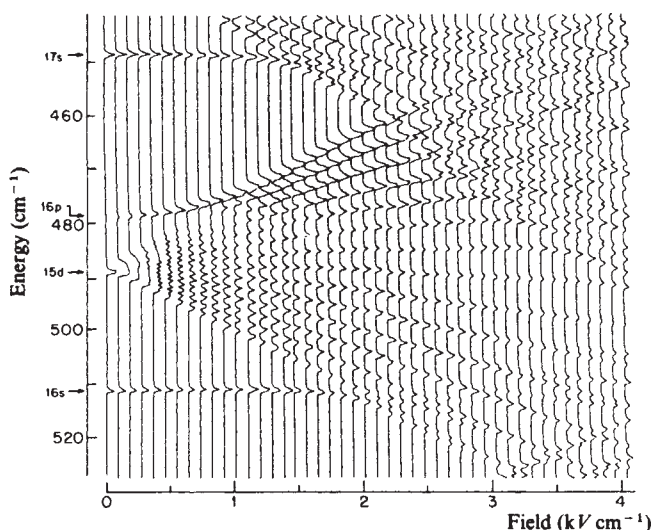


Fig. 4 The Stark spectrum of sodium (taken from ref. 18).

Spontaneous radiative transitions between Rydberg levels are very slow because the rate is proportional to the cube of the frequency, but stimulated transitions can be fast because the rate depends only on the strength of the radiation and the transition moment (square of the dipole matrix element) between the two levels involved. As the transition moment between two Rydberg states is very large (proportional to n^4), the rate is also very large and minute amounts of power are required to induce transitions. It is, therefore, fairly easy to do r.f. or microwave spectroscopy on these atoms^{12,13}. In fact, the matrix elements are so large that power broadening is difficult to avoid, and higher order transition processes (two or more quanta) occur readily.

There are many different kinds of r.f. experiments to be done. For example, r.f. spectroscopy of Rydberg atoms can produce high precision measurements of atomic parameters because of the very narrow linewidths (long lifetimes) of the transitions²⁰. One might measure fine structure splittings¹⁴, quantum defects, hyperfine interactions, or even fundamental constants by careful experiments on Rydberg atoms. Also, even in a moderately strong r.f. field, very high order processes (multi-photon) are easily observable²¹. Not only can these be used for spectroscopic investigations, but also for the testing of theories of ground state atoms in very strong optical frequency fields (high intensity laser light).

As transitions between Rydberg states can be induced by very tiny amounts of radiation, one must consider all possible sources in the description of such experiments. Although the power density of black-body radiation at room temperature is extremely small and is normally only considered in low temperature experiments, careful calculations and observations have shown that this radiation can appreciably shorten the lifetime of highly excited states by stimulated transitions²². One also expects that cooperative effects such as super-radiance and maser action may also be possible in a sufficiently dense population of Rydberg atoms.

The enormous transition moments of Rydberg atoms can be exploited to make sensitive detectors of low frequency radiation²⁰. As transitions can be detected by selective field ionisation, each quantum absorbed from an IR or microwave field produces a charged particle which can be readily detected. The result is a high efficiency, low noise, tuneable (via Stark effect), quantum detector of great interest in low temperature studies, astrophysics, communications, and so on.

A beam of atoms in Rydberg states can be thought of as transporting very low energy electrons at nearly uniform speeds. It is very difficult to make an electron beam monochromatic because of the thermal energy spread of the source, but Rydberg atoms divide the thermal energy between the electron and the very heavy nucleus so that the velocity distribution is very narrow. Collisions can be made either with Rydberg atoms directly on the target or by gently photoionising the highly excited electron and using it alone on the target. The resolution of electron collision studies on gases, beams and surfaces will be greatly enhanced by the use of Rydberg atom electron sources.

The previous discussion above has been restricted to Rydberg states of alkali metal atoms. It is possible to study the Rydberg states of molecules²³ or atoms with two or more valence electrons²⁴ (such as alkaline earths) and the added richness of the spectra provides new information. For example, after one electron is promoted to a Rydberg state, a second electron can be raised to one of the ion core's excited states. The atom now has too much internal energy to remain bound and autoionises, emitting an 'Auger electron'. This process may be enhanced by configuration interaction with another nearby state and precise studies can be made. A relatively new theoretical structure called multichannel quantum defect theory (MQDT) has been developed to describe these processes²⁵ which can be measured carefully and cleanly by Rydberg spectroscopy.

Conclusion

This discussion of the physics derived from Rydberg spectroscopy cannot be complete because of both the breadth of the field and its rate of development. There are a great many other topics which could not be included here because of space limitations. We have seen that many of the properties of Rydberg atoms can be accurately described by classical or very simple quantum mechanics, and that the apparatus for experimental studies is relatively small scale and inexpensive. Such simplicity makes the field very attractive, especially in view of the extraordinarily broad range of topics which can be investigated with these unusual atoms.

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ARTICLES

 $^{230}\text{Th}/^{234}\text{U}$ dating of travertine from the Bilzingsleben archaeological site**Russell S. Harmon*, Jerzy Głazek† & Krystian Nowak‡**

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$^{230}\text{Th}/^{234}\text{U}$ dating of travertine from Bilzingsleben, places an absolute age of $228,000 \pm 17,000$ yr BP on the hominid remains and artefacts at this important archaeological site. Associated floral and faunal fossil assemblages are of interglacial character indicating a depositional climate slightly warmer than at present. These two facts permit correlation of the Bilzingsleben deposits with 'Stage 7' of the marine oxygen isotope record and the penultimate interglacial observed in the European and North American speleothem records.

THE absolute age and stratigraphic position of most of the hominid-bearing deposits of western and central Europe are, at present, uncertain outside the $\sim 40,000$ yr BP limit of conventional ^{14}C dating. Many of these archaeological sites contain coeval inorganically precipitated calcium carbonate deposits which can, under certain circumstances, be dated by natural disequilibrium within the uranium decay series. For example, U-series dating techniques have been used by Turekian and Nelson¹ to date the travertine sequence at Caune de L'Arago, France, at $96,000 \pm 10,000$ yr BP, and by Schwarcz and Debenath² to obtain an absolute age of $146,000 \pm 16,000$ yr BP for skeletal remains in bed 11 of the l'abri Bourgeois-Delaunay rock shelter at La Chaise, France. Here we report $^{230}\text{Th}/^{234}\text{U}$ age determinations for the fossiliferous hominid- and artefact-bearing travertine deposits at the Bilzingsleben site in the German Democratic Republic and briefly discuss the stratigraphic and archaeological significance of this age determination.

The Bilzingsleben site (Fig. 1) is located in the northern portion of the 'Middle German Highlands' about 35 km north of Erfurt in the type-region of the northern European Pleistocene glacial stratigraphic sequence. The archaeological deposits, from which a fossil human skull was first described by von Schlotheim⁴ in 1818, occur in the Steinrinne travertine quarry

capping an isolated plateau of Triassic (Lower Keuper) marls and dolomites at 35 m above the Wipper River. This plateau has been partially dissected by river entrenchment with four distinct Pleistocene gravel terraces present at elevations of about 30, 20, 10 and 2–5 m within the river valley. Traditionally these terraces have been assigned, in descending order, to the Elsterian, Saalian, Warthian, and Vistulian glaciations⁵. The travertine and associated silts in the quarry fill a lake depression over the highest (30 m) terrace which was formed as the result of subsidence of a karst sinkhole. A schematic section through these deposits is shown in Fig. 2. Directly overlying the gravels of the 30 m terrace are 3–5 m of a slightly eroded silt with furrows in the erosional surface infilled with peatearth and gley soil. Above this is a 4–8 m thick travertine deposit consisting of the following lithologic sequence:

- (1) 30–50 cm of 'sandy' dense, light-brown travertine containing abundant fossils, artefacts and hominid remains;
- (2) 50–80 cm of lacustrine limestone;
- (3) 30–40 cm of 'soft' porous travertine;
- (4) 300–600 cm of 'dense', dark-brown travertine.

Previous studies^{6–8} have shown that calcium carbonate speleothems and travertines can be confidently dated by U-series methods if they contain trace amounts of U, but are free of

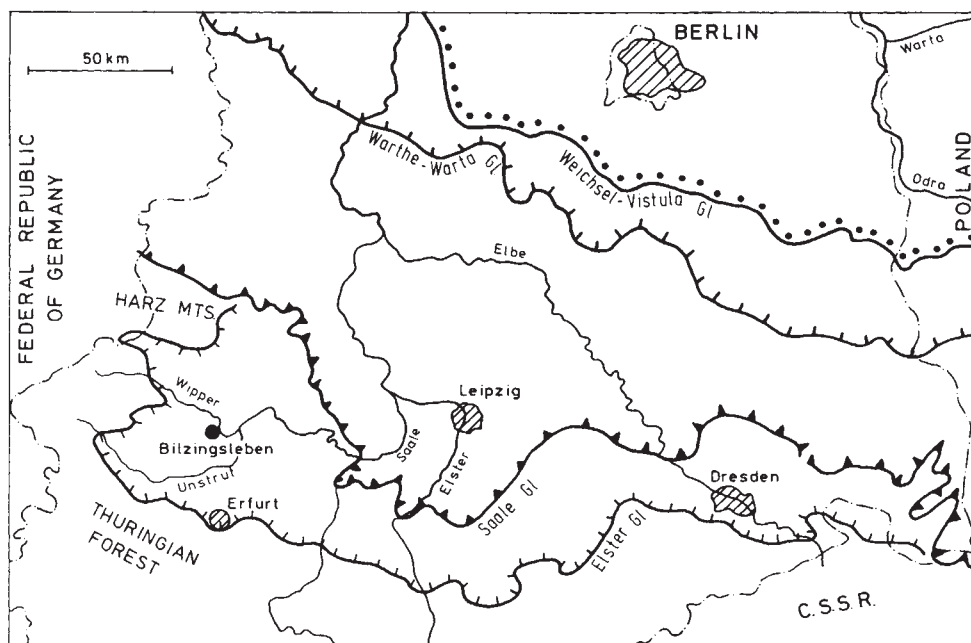


Fig. 1 The limits of continental glaciations in the southern part of the GDR (simplified after Cepek³) in relation to the Bilzingsleben archaeological site.

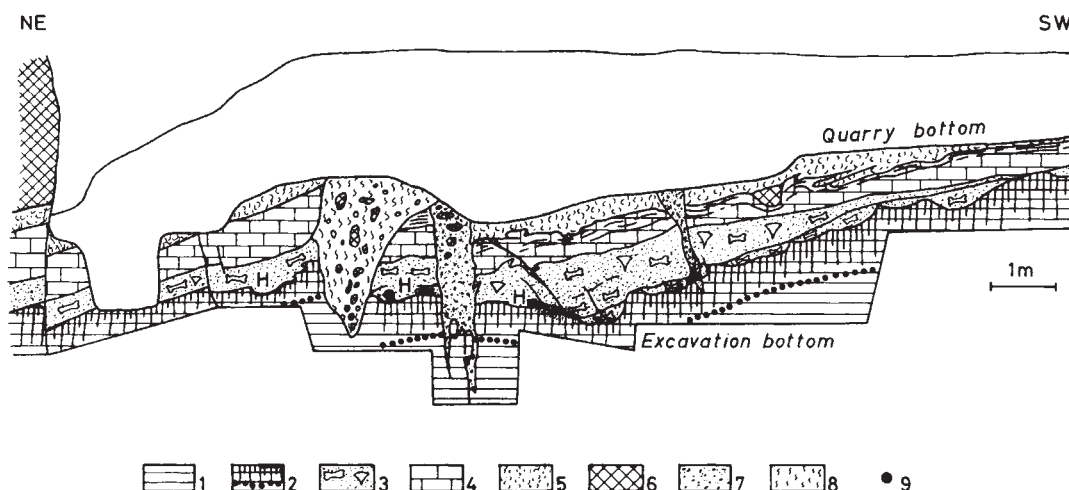


Fig. 2 Schematic cross-section of the travertine sequence in the Steinrinne Quarry near Bilzingsleben (simplified after Mania⁵). 1, Silts; 2, soil profile on silts (black on top: gley horizon, vertical dashes: peat earth, black spots in bottom: illuvial carbonate concretions); 3, 'sandy travertine'—hominid—artefact—and bone-bearing layer; 4, lake limestone; 5, soft travertine; 6, hard upper travertine; 7, fissure filling with travertine debris; 8, loess with travertine debris; 9, dated travertine sample; H, location of hominid bone fragments.

detrital Th or Pa. In such instances, absolute dating is possible within the time range ~5,000–350,000 yr BP provided the carbonate is dense and impermeable primary calcite or aragonite showing no signs of diagenetic alteration or post-depositional leaching. The Bilzingsleben travertine which we have dated by the $^{230}\text{Th}/^{234}\text{U}$ method occurs as a dense lens of light brown, micritic calcite within the fossiliferous lower, 'sandy' travertine horizon (Fig. 2). The travertine lens is neither a later (younger) intrusion into the bone-bearing unit, nor is it a detrital clast of unknown age. The stratigraphically younger upper, dark-brown travertine unit was not suitable for dating because it contained a substantial admixture of detrital sediments and iron oxides. Four crushed samples of about 50 g were dissolved in dilute nitric acid and chemically pure U and Th isolated by coprecipitation ion-exchange, and organic solvent techniques^{9,10}. $^{230}\text{Th}/^{234}\text{U}$ ages were calculated from yield and background corrected isotope activities as measured by α spectrometry—pulse height analysis. Analytical data and calculated ages are presented in Table 1. Note the high degree of internal consistency for U concentrations (4.3–5.0 p.p.m.), $^{234}\text{U}/^{238}\text{U}$ ratios (1.19 ± 0.02 – 1.24 ± 0.02), and $^{230}\text{Th}/^{234}\text{U}$ ratios (0.91 ± 0.02 – 0.93 ± 0.02) as well as the high $^{230}\text{Th}/^{232}\text{Th}$ ratios (34–202). This indicates that all ^{230}Th activity in the travertine was authigenic and that it had not been subject to post-depositional loss or addition of U, thus giving us confidence in the $^{230}\text{Th}/^{234}\text{U}$ age determination of $228,000 \pm 17,000$ yr BP.

The stratigraphic age of the Bilzingsleben deposit has been debated for over 50 years. Geological relationships indicate that

the travertine sequence and associated archaeological materials must be younger than Elsterian in age, but there is no other consensus. The interglacial character of the deposit is well established from the abundant floral and faunal fossil material contained in the hominid-bearing, lower travertine unit. Some 10 species of plants, 15 species of snails, and 12 species of vertebrates have been recognised^{5,11–14}. These are listed in Table 2. Ecological interpretation of this fossil assemblage suggests a forest environment with a climate slightly warmer than at present with mean annual temperature elevated by about 2 °C and without winter frost (ref. 15 and A. Sutcliffe, personal communication).

Our radiometric age of $228,000 \pm 17,000$ yr BP permits direct correlation of the Bilzingsleben travertine with 'Stage 7' of the marine foraminiferal oxygen isotope record¹⁴ and the penultimate interglacial period in the North American¹⁷ and European^{18,19} speleothem chronologies (Fig. 3). However, placement of the deposit within the north-central European glacial stratigraphic sequence is more problematic. Although contemporaneity with the Lublin Interglacial in Poland (dated at $245,000 \pm 45,000$ yr BP) (ref. 20) and the Odintsovo (Kaydaki) Interglacial in the western USSR (dated at $227,000 \pm 28,000$ (ref. 21) and $236,000 \pm 25,000$ (ref. 12) yr BP) is suggested on the basis of thermoluminescence ages for these deposits, further correlations of this kind are not possible because of a lack of absolute age data. At some time the Bilzingsleben deposit has been assigned to the Elster–Saale (Holstein)^{5,15,23,24}, Saale–Warthe²⁵, or Warthe–Weichsel (Eem)^{13,26} interglacial periods.

Table 1 U concentrations, isotope activity ratios, and calculated age for the Bilzingsleben hominid-bearing, lower travertine unit

Sample	Description	[U] (p.p.m.)	$\frac{[^{230}\text{Th}]}{[^{234}\text{U}]}$	$\frac{[^{234}\text{U}]}{[^{238}\text{U}]}$	$\frac{[^{230}\text{Th}]}{[^{232}\text{Th}]}$	Age (yr BP)
373	Bulk sample	4.4	0.92 ± 0.02	1.22 ± 0.02	202	$228,000 \pm 17,000$ –12,000
374	Fine fraction (270 mesh)	4.3	0.93 ± 0.02	1.24 ± 0.02	34	$234,000 \pm 18,000$ –13,000
375	Intermediate fraction (70–270 mesh)	4.5	0.91 ± 0.02	1.19 ± 0.02	75	$223,000 \pm 16,000$ –11,000
376	Coarse fraction (>70 mesh)	5.0	0.91 ± 0.02	1.21 ± 0.02	76	$222,000 \pm 16,000$ –11,000
	Average		0.92 ± 0.02	1.22 ± 0.02	97	$228,000 \pm 17,000$ –12,000

Table 2 Fossil flora and fauna from the Bilzingsleben hominid-bearing, lower travertine unit (after Mania⁵, Wiegers¹¹, Vent¹², Wohlstedt¹³ and Wüst¹⁴)

Plants	Snails	Vertebrates
<i>Buxus sempervirens</i>	<i>Vitina elongata</i>	<i>Castor fiber</i>
<i>Picea excelsa</i>	<i>Zonites verticillus</i>	<i>Trogontherium</i> sp.
<i>Rubus</i> sp.	<i>Patula solaria</i>	<i>Glis glis</i>
<i>Acer pseudoplatanus</i>	<i>Fruiticola umbrosa</i>	<i>Canidae</i> sp.
<i>Betula pubescens</i>	<i>Dibothrium bidens</i>	<i>Ursus arctos</i>
<i>Corylus avellana</i>	<i>Cepaea nemoralis</i>	<i>Felidae</i> sp.
<i>Quercus</i> sp.	<i>Clausilia pumila</i>	<i>Palaeoloxodon antiquus</i>
<i>Salix cinerea</i>	<i>Clausilia tumida</i>	<i>Mammuthus primigenius</i>
<i>Rhododendron</i> sp.	<i>Clausilia filograna</i>	<i>Equus caballus</i>
<i>Fraxinus excelsior</i>	<i>Orcula doliolum</i>	<i>Dicerorhinus mercki</i>
	<i>Isthmia claustratis</i>	<i>Cervus elaphus</i>
	<i>Vertigo moulinsia</i>	<i>Caproeolus capreolus</i>
	<i>Vertigo pusilla</i>	
	<i>Azeca schulziana</i>	
	<i>Belgrandia germanica</i>	

It is generally accepted that the Eemian interglacial deposits of Denmark are correlative with 'Stage 5' of the marine oxygen isotope record so that the Bilzingsleben deposit cannot be of last interglacial age. Similarly, a Holsteinian age is ruled out if the arguments of Kukla²⁵ that the Saale ground moraines are at least of 'Stage 8' (and more likely of 'Stage 10') age are accepted. Cepek²⁵ has argued that the Rügen deposits represent an interglacial period between the Saalian and Warthian glacial events, but both the position and nature of this deposit are controversial. The Rügen deposit may, in fact, be correlative with the Bilzingsleben deposits, but there is insufficient evidence to justify such a claim. Therefore, we propose to establish the Bilzingsleben deposit as the type section for the penultimate interglacial period in north-central Europe because the age and interglacial character of the deposit are now firmly established.

Hominid remains from Bilzingsleben were noted as early as 1818 (ref. 4). Since 1969 excavation by the Landesmuseum für Vorgeschichte, Halle a. Saale has produced over 60,000 flint artefacts as well as rock, bone and antler tools⁵. Most of this material is without typology although some Mousterian and Tayacian artefacts have been recognised leading Toepfer²⁶ to assign a Pontiano-Mousterian character (similar to that of

Saccopastore) to the Bilzingsleben artefacts. More recently a Clactonian character for the artefacts has been suggested⁵. Recently discovered hominid bone material^{5,23,24,28} consists of os occipitale, os frontale and one molar. Taxonomic analysis by Stringer *et al.*²⁹ suggests that these hominid fragments represent an archaic form of Middle Pleistocene *Homo sapiens*, although exhibiting some more primitive morphological features which have led Vlček³⁰ to classify them as a new subspecies of *Homo erectus*. The fossils are similar in form to those from Vértesszöllös, bear some resemblance to those from Petralona, Steinheim, and Swanscombe, but are distinctly less evolved than the hominids at Saccopastore, La Chaise and Ehringsdorf²⁹. The samples of the lower travertine unit dated here are directly associated with the Bilzingsleben fossils and artefacts (Fig. 2) and thus an age of 228,000 $\pm 17,000$ yr BP can be assigned to this archaeological site.

This age determination has important implications as regards Middle Pleistocene hominid evolution. It is widely assumed that *Homo erectus* evolved into *Homo sapiens*, but the fossil evidence is equivocal. If Vlček's³⁰ contention that the Bilzingsleben fossils represent a new subspecies of *Homo erectus* and the somewhat dubious U-series ages of $\sim 200,000$ – $220,000$ yr BP for the Ehringsdorf lower travertine unit^{8,27,31} are accepted, then it must be concluded that *Homo erectus* and *Homo sapiens* coexisted in central Europe during the penultimate interglacial period or that our present concepts of variation within these hominid species have to be considerably revised. If not, then morphological differences between the primitive hominid form of Petralona, the archaic forms of Bilzingsleben and Vértesszöllös and the more advanced forms of Ehringsdorf, La Chaise, and Saccopastore could represent progressive transitions within a single lineage of *Homo sapiens*. Between-site morphological differences (for example compare Swanscombe and Steinheim with Bilzingsleben and Vértesszöllös) would then be explained in terms of both phyletic change and sexual dimorphism within individual populations which may be superimposed on temporal, geographic and population variations²⁹.

Nevertheless, from the absolute age data presented here, and similar archaeometric studies from France^{1,2} and Greece⁸, it is evident that the archaeological correlations predominantly based on the antiquated concept of only four Pleistocene glaciations must be revised. It is evident that (1) many Middle Pleistocene archaeological deposits (such as that at Bilzingsleben) assigned on meagre and equivocal field evidence to a particular interglacial period, may be miscorrelated and, in fact, represent

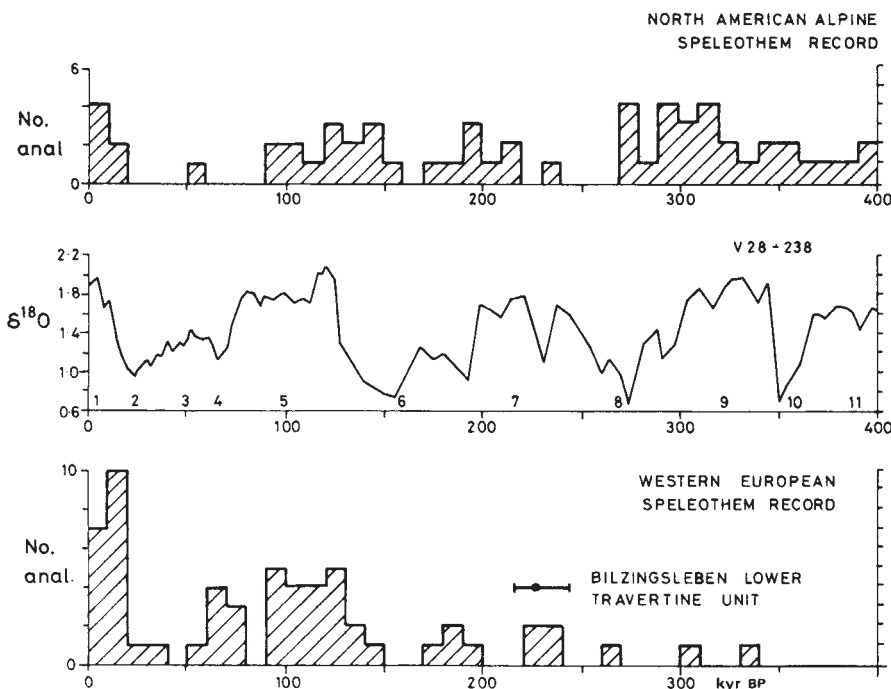


Fig. 3 Comparison of the Bilzingsleben age with the marine foraminiferal oxygen isotope record of Shackleton and Opduke¹⁶, and the North American speleothem chronologies of Harmon *et al.*¹⁷, Atkinson *et al.*¹⁸ and Harmon¹⁹ establishing a penultimate interglacial age for deposition of the Bilzingsleben travertine.

an interglacial event not necessarily recognised in the European terrestrial glacial stratigraphic record, and also that (2) supposed Holsteinian deposits may belong to two or more interglacial periods. Further archaemoetric studies of the type described here are required to resolve both of the ambiguities relating to the correlation of European prehistoric cultures and industries (for example, the three different ages for the British Acheulian cited by Collins³²) and are necessary to clarify further the pattern of hominid evolution in Europe (where there is increas-

ing evidence to suggest that *Homo erectus* and *Homo sapiens* may have coexisted during the Middle Pleistocene).

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The crack–seal mechanism of rock deformation

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Many naturally deformed crustal rocks contain mineral-filled extension veins. The crystals making up the vein filling often show a fibrous habit and seem to be built up by a succession of 'crack–seal' increments: the elastically deforming rock fails by fracture, and the walls of the open micro-crack are sealed together by crystalline material derived by pressure solution in the rock matrix.

MANY of the rocks deformed by natural tectonic processes that have occurred in the upper 30 km of the Earth's crust show the development of more or less regular systems of extension cracks and fissures, the fissure spaces filled with silicate or carbonate minerals. The mechanisms by which such veins develop is of special interest to our understanding of crustal deformation. In many orogenic regions extension fissures can apparently give rise to large finite elongations and they can lead to finite strains of several per cent, locally exceeding 50%. The evidence presented here suggests that many, if not most, of these extension veins are formed by an accretionary process involving the formation of a narrow fracture followed by filling of the open space by crystalline material, a mechanism termed 'crack–seal'. Geometric features implying this mechanism have been most commonly observed in rocks deformed at metamorphic grades up to upper greenschist facies in environments where stress-induced chemical transfer (pressure solution) of materials seems to be relatively common. The minerals appearing in the vein fillings cover a wide range of mineral species (for example, quartz, calcite, feldspar, actinolite, biotite, and chlorite).

Early stages of vein formation: structure of narrow veins

The first example of the crack–seal process to be described here is taken from pisolitic ironstones of Lower Tertiary age situated

in the frontal folds of the Morcles nappe, one of the important units of the Helvetic nappes, near the village of Champéry, Valais, Switzerland.

The rock is a dense ironstone with sub-spherical, well layered chloritic ironstone pisolites up to 6.0 mm in diameter, and with scattered, isolated, rounded to sub-angular grains of clastic quartz. Figure 1a shows a single crack 45 µm wide passing through matrix, quartz grains and chamosite. The two sides of the crack have been joined together with silicate material; the separate fragments of quartz are sealed together with clear quartz in optical continuity with the broken, inclusion-rich quartz parent, whereas the chamosite grains and ironstone matrix are sealed with fibrous green chlorite. Figure 1b illustrates a vein built up by repetitions of this cracking and sealing process. Eleven small veinlets, each of ~45 µm width, group together to form a compound vein which in the rock specimen appears to the naked eye as a single white vein 0.5 mm in width. The optical control of the successively formed veins by the separated pieces of wall rock is very clear. In the thin sections the successively sealed veinlets give rise to very characteristic 'wing'-like slots of new quartz which in three dimensions have a 'Saturn ring'-like form (Fig. 2). It seems that successive cracks took place along the vein–matrix contacts of a previously sealed crack system, that often a small amount of wall rock material was detached during each successive crack, and that this wall material was preserved as an inclusion trail between the cracks marking successive incremental extensions (Fig. 1c). The cracks

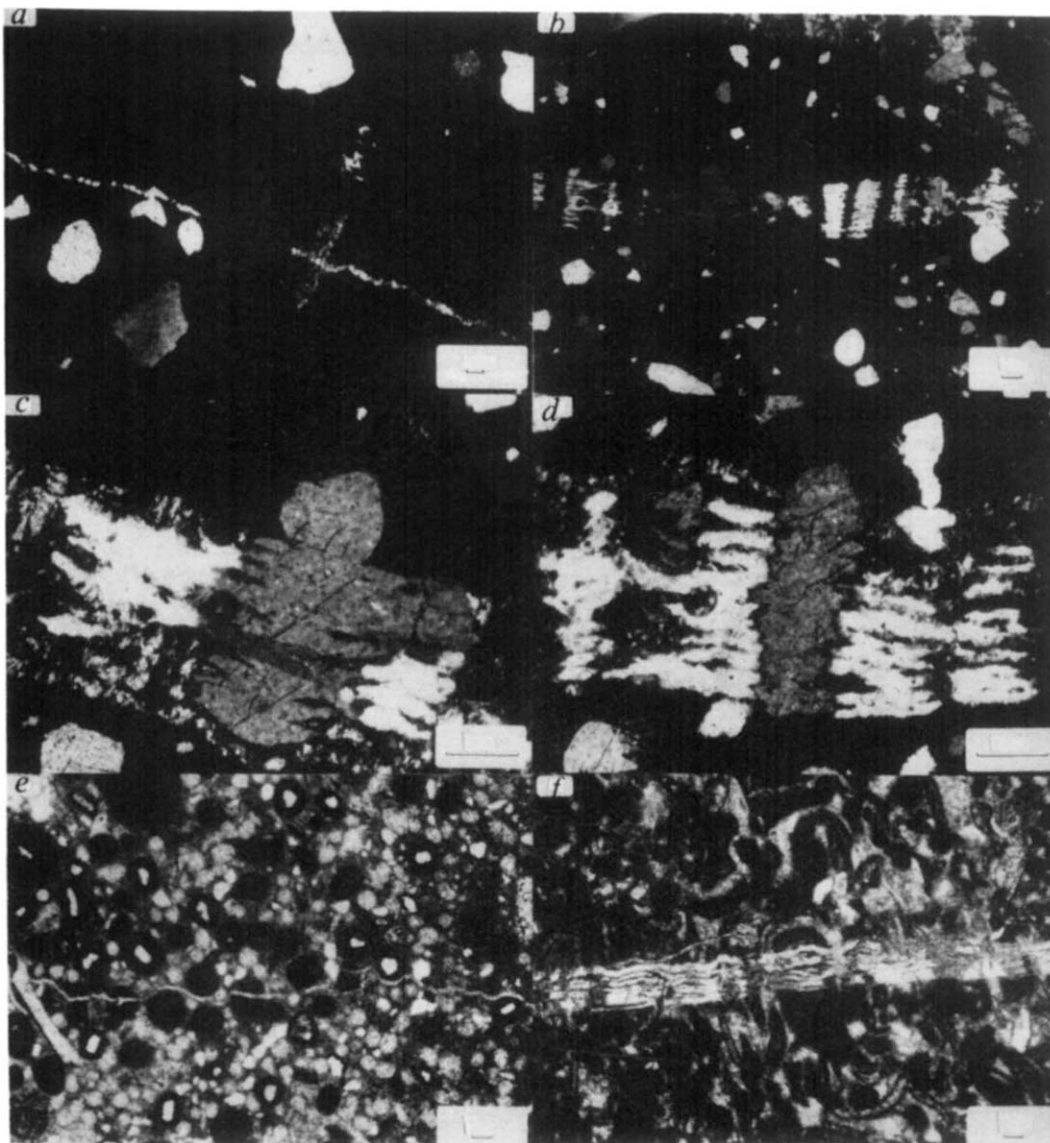


Fig. 1 *a-d*, Thin sections of crack-seal veins in a sandy pisolitic ironstone: *a*, single quartz-chlorite filled crack passing through clastic quartz and chlorite grains; *b*, an array of several small crack-seal veinlets making up a single compound vein; *c*, detail from *b* showing inclusion bands parallel to the main vein wall, the general optical continuity of vein filling with the parent grain in the vein wall, and one crack-seal band (darker grey) slightly misorientated to the other bands; *d*, characteristic 'sawtooth' contacts between elongated fibrous quartz crystals. *e, f*, Thin sections of calcite filled crack-seal veins in oolitic and organic clastic limestones. All scale bars, 100 μm .

occur along the previous vein contacts because this is the overall mechanically weakest surface in the rock.

Although successively grown new quartz strips are generally in optical continuity with the parent quartz, occasionally a misfit of *c*-axis of up to about 3° is seen (Fig. 1*c*). These misfits probably occur because the new growth in a veinlet may be guided by a slightly misorientated seed fragment of broken wall. Where the successive cracks pass along the margin of a quartz grain the inclusion trails so derived are rich in dark iron-rich matrix components, whereas where a crack passes through the centre of a quartz sand grain, the wall-derived inclusions consist mostly of quartz and are visually not so striking. However, the margin of an internally broken quartz grain which has undergone successive fracture and sealing shows a very characteristic saw-tooth form (Fig. 1*d*).

The second example of the crack-seal mechanism is taken from a quite different rock type. The rock is a bioclastic limestone of Jurassic age from the Clariden region of central Switzerland. The limestone sequence is in part oolitic, and in part consists of fragments of shells, crinoids and other organic debris. Figure 1*e* shows a single extension crack passing through an oolitic limestone. The crack sometimes passes directly through the ooids and the matrix, and in places it seems to have been guided by the mechanically weaker boundaries of the oolitic particles. It has been filled with small crystals of clear, crystalline calcite which grow in optical alignment with small

calcite crystals making up the ooids and their matrix. Figure 1*f* shows a succession of calcite sealed microcracks separated by very narrow zones of inclusions of screens of wall rock material, producing an overall vein structure which is remarkably similar to that of the sandy ironstone of the first example. It is especially remarkable that the width of the microveins in the two examples is almost the same, and that the optical orientations of the calcite crystals passing through the composite veinlet aggregate is continuous across the veins, as was the optic orientation of the quartz joining the fragments of broken sand grains.

Crack-seal structure in large veins

The third example of the crack-seal process is a very spectacular one, and illustrates especially well the way that the seal material is added on the walls of the previously formed crack. It comes from highly deformed ferruginous limestones of middle Jurassic age from the Windgällen situated in the autochthonous cover rocks of the Aar Massif of central Switzerland. The limestones contain chamosite oolites and are sufficiently rich in haematite and magnetite to have been mined for their iron content in the latter part of the nineteenth century even though they are situated at an elevation of 2,400 m. The tectonically formed veins are found along planes sub-perpendicular to the direction of maximum finite stretching as determined from the shapes of the deformed oolites^{2,3}. The veins (Fig. 3) consist of fibrous intergrowths of quartz and calcite, the fibres being orientated

perpendicular to the walls of the veins. The veins have the characteristics of antitaxial vein growth⁴—that is, the crystals grow from an approximately centrally located inclusion-rich zone (the median surface) towards the two walls. Some of the individual crystal fibres become larger towards the walls, presumably because they were more favourably orientated for growth than their neighbours.

The most remarkable feature of these antitaxially formed veins is the presence of very regularly orientated lines of solid inclusions, mostly consisting of tiny crystals of chlorite and calcite arranged sub-parallel to the walls of the vein at distances of about 12 μm apart. These lines of inclusions are termed inclusion bands (Figs 3, 4b, 4c). The undulations seen in one inclusion band are more or less parallel to those in the adjacent bands, but the undulations gradually change shape from inclusion bands in the vein centre to those near the vein wall (Fig. 4b). The inclusion bands close to the wall are parallel to the vein-wall contact (Figs 3, 4b). As well as a correspondence of shape between adjacent inclusion bands there is also a striking correspondence in inclusion composition and grain shapes of the individual crystals making up the bands. This is particularly noticeable within calcite inclusions; these form lines of separate, optically parallel and shape related grains aligned sub-perpendicular to the vein wall, termed inclusion trails (Figs 3, 4c, 4d, 4f). In many instances these small grains can be shown to be completely enclosed in the larger fibrous grains of the main host vein crystal, and under the microscope it can be proved that there could have been no direct physical connection with some parent grain located outside the plane of the thin section (such as occurs when a section passes through the edges of the Saturn-ring structure of the crack-seal veins of our first example, shown in Fig. 2). In a few inclusion trails the individual inclusions come very close to each other, and some do form series of more or less continuously linked crystals (Fig. 4d). The composition of crystal components of adjacent inclusion bands change sympathetically (Figs 3, 4c). An inclusion band made up of chlorite particles will change along its length to one made up of calcite crystals, and adjacent bands show identical changes in composition (Figs 4c, 5). The compositional changes along adjacent bands always occur along a line parallel to the inclusion trails. The mineral component changes can be related to the composition of the vein-matrix wall; the inclusion trails of calcite always

root on to the normal granular carbonate of the rock wall, whereas the chlorite inclusion trails always root on to a wall made up of the broken surface of a green chlorite ooid. The trails of optically parallel calcite inclusions lead to an optically identically orientated clear calcite crystal growing on the vein-matrix wall and which itself is optically aligned to a more opaque carbonate fragment of the matrix (Figs 4f, 5).

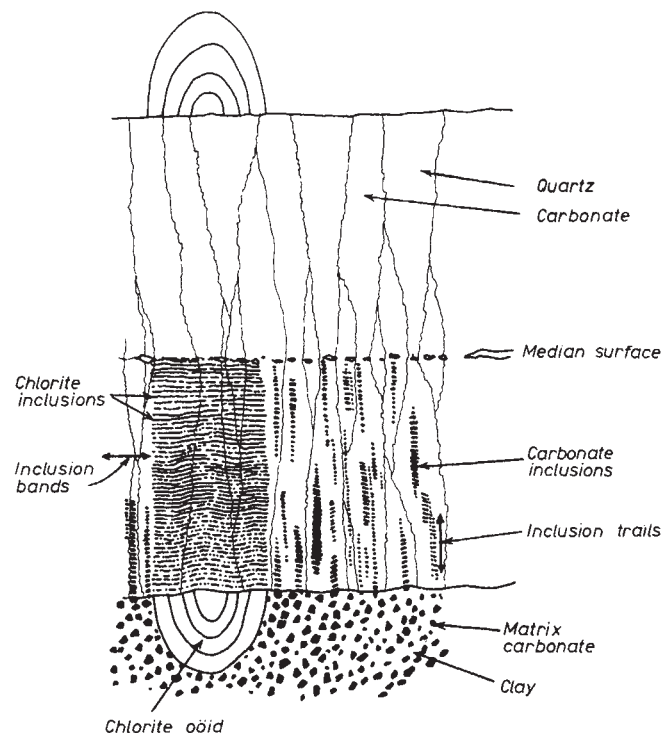


Fig. 3 Schematic diagram to show the main features of quartz-calcite tectonic veins in the oolitic limestone of the Windgällen. The vein on both sides of the median surface shows inclusion bands and inclusion trails, but these have been omitted on the upper side of the median surface so as to show the general shapes of the main vein-forming minerals.

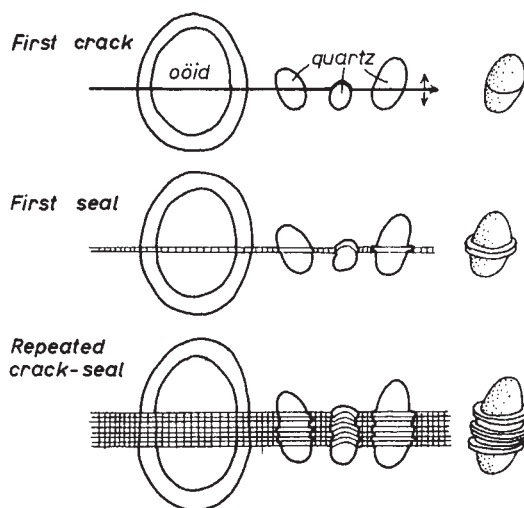


Fig. 2 Schematic development of crack-seal veins. The left-hand side shows the appearance of successive crack-seal veins in cross-section, while the right-hand side illustrates what is believed to be the three dimensional Saturn-ring structure deduced from successive two dimensional thin sections.

The relationships of the large fibrous quartz and calcite crystals forming the main vein filling to each other and to the small crystal inclusions is in main geometric features simple, but, in detail, quite complex. In general the grain-to-grain contacts of the larger crystals are sub-parallel to the inclusion trails (Figs 3, 4d). Calcite and chlorite inclusion trails and bands can be formed in both quartz and calcite forming the larger vein-crystals (Fig. 4f, calcite in quartz, and calcite in calcite in the dark centrally located grain; Fig. 4c, chlorite in quartz and chlorite in calcite). The chemical control on the composition of the inclusion crystals seems to be exerted by the composition of the matrix at the vein-matrix wall and not by the composition of the main vein crystals. Although the contacts of large vein-forming crystals are generally almost parallel to the inclusion trails in several examples the inclusions forming a trail transect these contacts passing from one large main vein crystal to another (Figs 3, 4d, 5). The quartz-quartz, quartz-calcite and calcite-calcite contacts of the main vein crystals often show a saw-tooth shape (Figs 4b, 4d, 5), the dimensions of the irregularities being the same as those of the inclusion band spacing. Although the large vein-forming crystals linking across the inclusion bands are generally optically continuous (Figs 4e, 4f), some slight irregularities do occur in the optical parallelism between certain inclusion bands. Variation in *c*-axis orientation

of up to 3° gives rise to a gently undulating dark-line polarisation colour appearance under crossed polars (Fig. 4c), and in a few instances slight variations of *c*-axis orientation in a single large individual crystal seems to be the result of plastic deformation after crystallisation (Fig. 4b).

The remarkable geometric features of these veins have been described in detail because they seem to provide a key to the understanding of vein-growth by the crack-seal mechanism. The inclusion band and inclusion trail structure seems to be best explained with recourse to progressive incremental cracking along the vein-matrix contacts, followed by a sealing together of the crack walls by crystalline material precipitated from a fluid phase. The successive microcracks were filled with fluids containing components in solution which were chemically closely related to the components forming the main body of the rock, and which were probably derived by local chemical solution and transfer (pressure solution). Some of these components (Ca^{2+} , CO_3^{2-} , Mg^{2+} , Fe^{2+} , Fe^{3+} , Al^{3+} , Si^{4+}) were then precipitated by syntaxial overgrowth on the crystals making up the microcrack walls as new formed calcite and chlorite. Some of the wall rock calcite crystals were favourably orientated for rapid growth (see, for example, Fig. 5, calcite in trail C_{14}) and could form a complete wall to wall link between the two sides, whereas others (Fig. 5, crystals in trails C_{11} , C_{12} , C_{13} , C_{15}) did not grow so fast and were therefore cut off by other faster growing crystal components before they could form a cross-link. The main crystal components which make up the continuous long fibres

(Fig. 5, quartz Q_1 , Q_2 , Q_3 and calcite C_1) were nucleated in the first formed crack, and because newly forming cracks always develop along the mechanically weak contacts of earlier formed veins, successive additions of new material lead to fibrous growth of these crystals away from the initial vein (antitaxial growth). These crystals can only grow sideways parallel to the vein at the expense of less favourably growth orientated neighbours. These space competing neighbours might be of the same mineral species (Fig. 5, quartz grain Q_2 growing at the expense of grains Q_1 and Q_3) or of a different species (calcite grain C_1 growing at the expense of quartz grain Q_3 and including unfavourably orientated calcite C_{15} on an inclusion trail). The crystallisation of the main vein component usually leads to a complete solid seal across the walls, but occasionally one finds remnants of fluid phases preserved as small fluid-gas bubble inclusions or negative crystal fillings. The fact that crystallisation of solid proceeds to form a complete wall-to-wall seal implies either that fluid containing the source of solid in solution must continually flow through the fissure, or that there is a continual replenishment of solid phase from the vein walls into the fluid phase occupying the progressively filling fissure. Once the sealing of the walls of the microcrack is complete, tectonic stresses are able to be transmitted across the vein and its walls once more. The intensity of these stresses builds up until a critical level of stability is achieved. The vein matrix contact then fails and a new fracture space is developed whose dimensions are controlled by the elastic recovery of the stressed walls. The

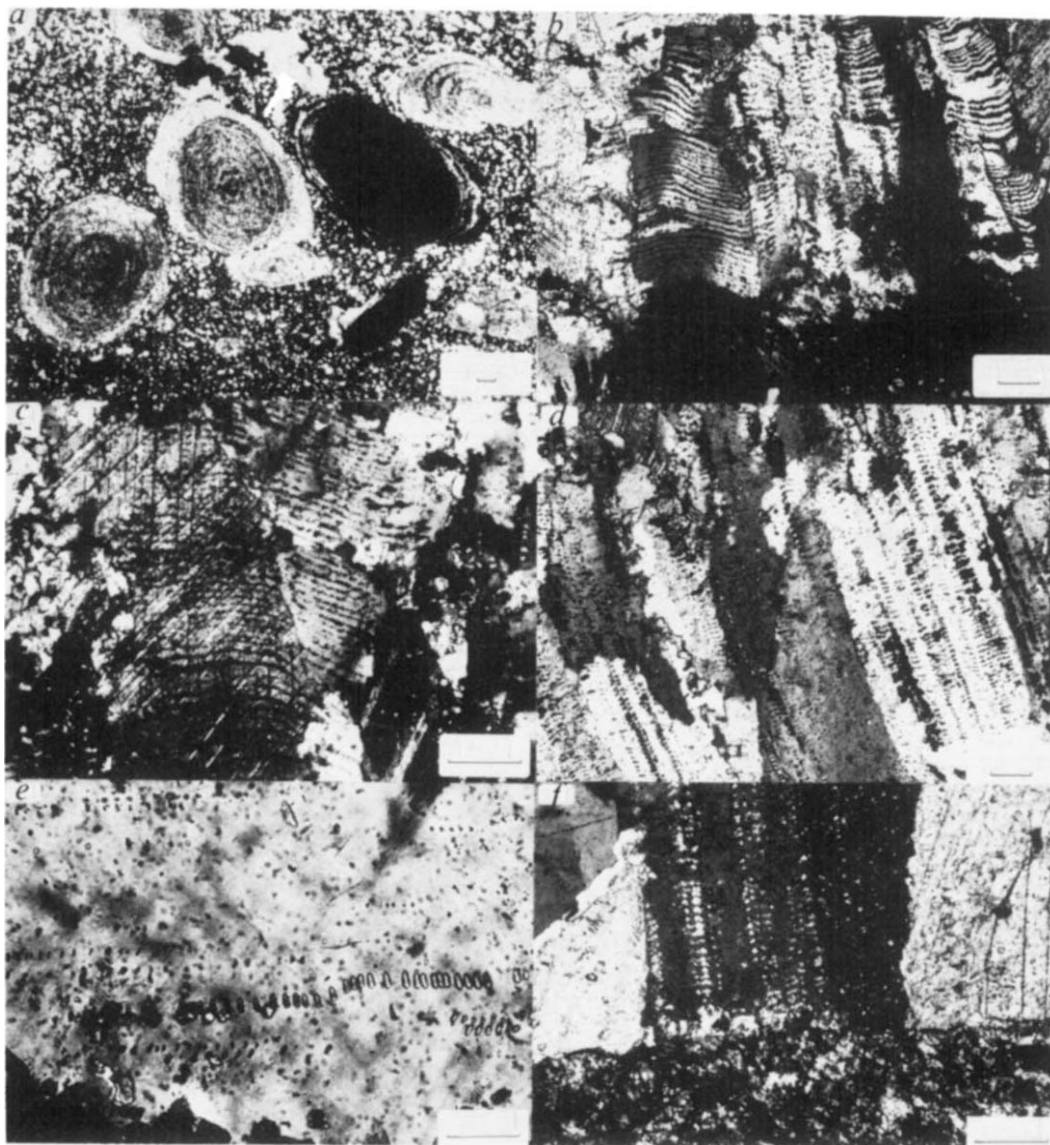


Fig. 4 Thin sections of crack-seal veins from the Windgällen, all taken with crossed polars. *a*, The chamosite ooids with characteristic concentric structures in a dark iron-rich matrix containing pale carbonate grains; *b*, inclusion bands parallel to the vein wall—chlorite inclusion bands occur where the wall of the vein is occupied by an ooid, whereas calcite inclusion bands are found where normal matrix carbonates are found along the vein walls (see Fig. 5); *c*, inclusion bands passing through large calcite and quartz crystals and changing in composition from chlorite components (left to right); *d*, inclusion trails of calcite passing through quartz fibres; *e*, detail of calcite inclusion trails in quartz showing the remarkable shape similarity of the individuals making up an inclusion trail; *f*, calcite inclusion trails linking on to wall rock calcites with the same optical orientation. All scale bars, 100 μm .

Fig. 5 Schematic diagram showing the main features of the inclusion band and inclusion trail structures in the Windgällen veins. Calcite crystals with line ornament, quartz crystals stippled.

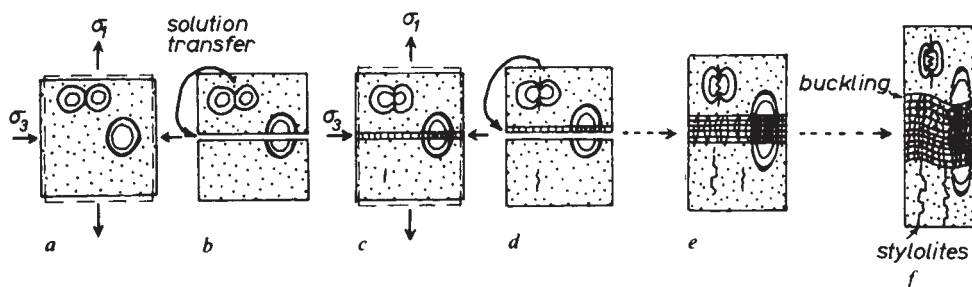
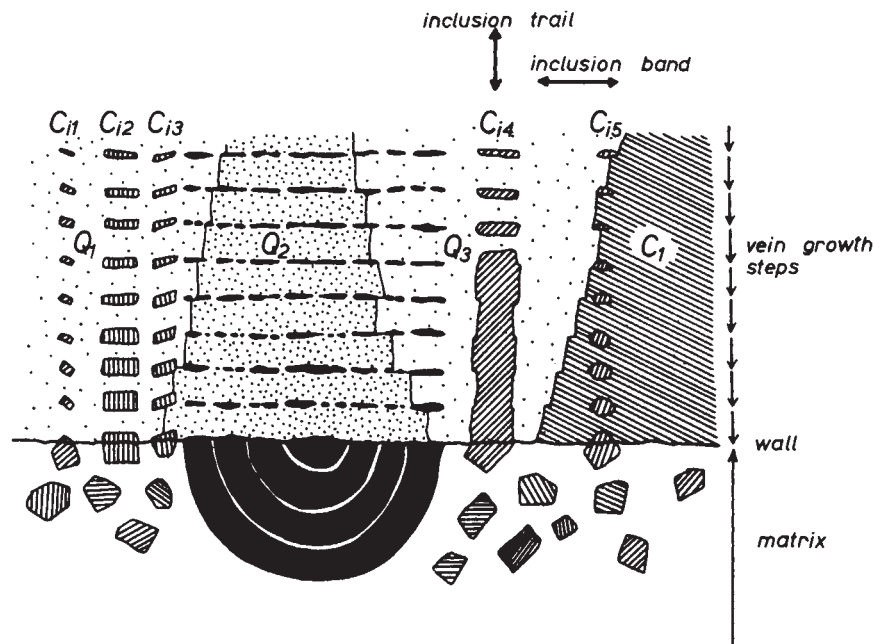


Fig. 6 Diagram showing the sequence of events leading to elongation of a rock by the crack-seal mechanism: *a*, elastic strain accumulation (σ_1 and σ_3 are greatest and least principal tensile stresses); *b*, development of first fracture and release of elastic strains in matrix, solution transfer

of material from walls into microcrack; *c*, seal of vein walls and further elastic strain accumulation; *d*, development of second crack and release of elastic strains in vein walls, further solution transfer with development of stylolitic surfaces; *e*, repeated crack-seal microcracks forming a compound vein; *f*, large finite shortening leading to vein buckling and the formation of stylolites both outside and inside the compound veins.

process of sealing the walls is repeated, and the crack-seal cycle is repeated indefinitely providing that sufficient tectonic stress accumulates for cracks to develop or that sufficient material is available in the solution which fills the cracks to seal the walls together. The Windgällen veins described here show more than 500 increments in a total vein width of 7.5 mm.

Conclusions

Figure 6 gives a review of the sequence of events by which a large overall extension by the crack-seal mechanism can be achieved. Tectonically induced elastic strains reach a critical level, and then fracture occurs (Fig. 6, *a* and *b*). The mechanism which brings about tensile fracture is probably that termed hydraulic fracturing⁵⁻⁷. There is strong evidence from the later precipitation of materials in the fracture that a fluid phase was present during fracturing, and it is well established that tensile fracturing can be initiated when the fluid pressure equals the tensile strength of the rock. Fluid-filled open microfractures are formed which are later filled with crystalline material derived by pressure solution in the rock matrix and chemical transfer of this material into the low pressure fluid-filled space (Fig. 6*c*). The

process is repeated and new material is added antitaxially so that the vein walls of the initial vein accumulate new material. The outward growth occurs because, statistically, there are equal chances of new cracks forming and subsequent new sealing material developing on both vein-matrix walls (Fig. 6*e*). At high finite strains (say greater than 10% shortening) the veins themselves have to shorten. Vein shortening is accomplished by buckling or by the development of tectonic stylolites along the grain boundaries of adjacent previously crystallised fibres (Fig. 6*f*).

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The use of synchrotron radiation in time-resolved X-ray diffraction studies of myosin layer-line reflections during muscle contraction

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Experiments on striated muscle have been carried out at the EMBL Outstation at DESY, Hamburg, using the electron-positron storage ring DORIS as a high-intensity X-ray source. The low-angle reflections from the myosin cross-bridges could be recorded more than 1,000 times more rapidly than with the best conventional X-ray sources, and could be followed during contraction with a time resolution of 10 ms.

DETAILS of the molecular mechanism which produces the sliding force between actin and myosin filaments during muscle contraction have proved difficult to establish. It has long been suggested^{1,2} that the force is developed by moving projections or cross-bridges on the myosin filaments which may undergo a cyclical process of attachment and detachment with sites on the actin filaments, but direct structural evidence for this mechanism is inherently difficult to obtain. Surviving muscles give informative low-angle X-ray diffraction diagrams³⁻⁵ which, in principle, can reveal details of cross-bridge behaviour during contraction. However, the use of this approach has previously been restricted by the long exposure times needed to record the patterns on film, although much useful information has been obtained⁴⁻⁹. Recently the use of increasingly efficient and sophisticated X-ray counters and electronic data handling systems has made it possible to carry out time-resolved measurements on the stronger equatorial and meridional reflections from striated muscles with a time resolution in the 10 ms range^{10-16,28,29}. This is the time scale required to make useful comparisons with normal tension development and decay in the muscles commonly used (for example, frog sartorius), but is too slow to study faster transient processes. Moreover, the off-meridional pattern of myosin layer-lines, which arises from the helical arrangement of the cross-bridges, is too faint to be recorded with adequate time resolution, even using very long contraction series, if normal laboratory-based X-ray sources are used.

For this and analogous reasons, there has recently been considerable interest in the use of synchrotron radiation as a high-intensity X-ray source for diffraction experiments^{17,18}. We report here some observations made at the EMBL Outstation at DESY, Hamburg, using the electron-positron storage ring DORIS.

Use of a two-dimensional TV detector

In our initial experiments we were concerned to establish whether or not the loss of layer-line intensity, which had earlier been observed (using film and laboratory X-ray sources) to occur during a very long series of tetanic contractions, was a genuine effect occurring during an unfatigued contraction in a freshly dissected muscle. We used a frog sartorius muscle, maintained in an oxygenated Ringer solution in the usual manner^{4,8} and stimulated directly by platinum electrodes lying along its length on either side. Tension was measured and recorded electronically. A focused and monochromatised X-ray beam was produced from the incident radiation from the storage

ring by a camera designed by Rosenbaum and Harmsen¹⁹, comprising an eight-element, totally reflecting glass mirror of total length 160 cm and a germanium curved crystal monochromator. With this apparatus, a good-quality layer-line pattern from muscle could be recorded on film in a few minutes, compared with about 24 hours for a similar quality pattern using a high-power rotating anode X-ray tube. In the actual experiments, however, the pattern was recorded on an X-ray image intensifier-TV detector^{20,21}, as this enabled good layer line data to be collected with a total exposure time of only 1 s. The two-dimensional nature of the detector had the great advantage that the whole of the X-ray pattern was available for inspection and measurement, and indeed the myosin layer-lines could be viewed directly in 'real time' on a monitor integrating over about 1 s. In this way, it was possible to see the pattern disappear as the muscle was stimulated tetanically for 1 or 2 s, and then reappear as soon as stimulation ceased. However, when collecting time-resolved data, only the pattern corresponding to one particular

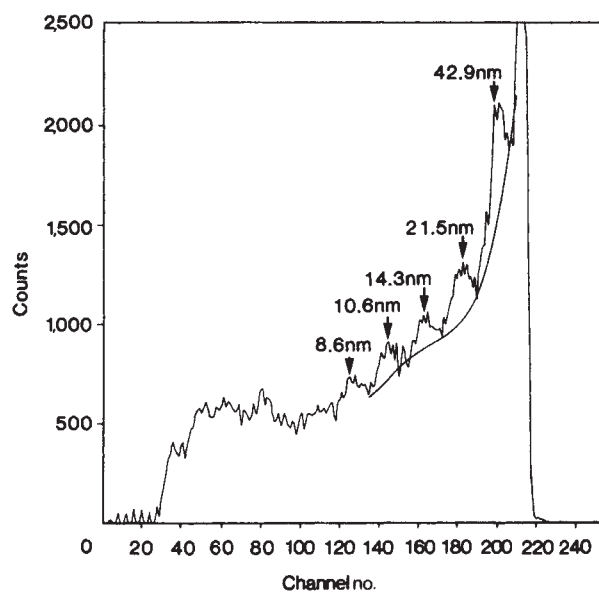


Fig. 1 Layer-line pattern from resting frog sartorius muscle at 10 °C recorded on the position-sensitive counter. Background fitted using a fourth order polynomial; total exposure time 3 s. Layer-line spacings are indicated.

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'time-slot' could be recorded in a given series of experiments because of readout time limitations with this type of detector.

To investigate the behaviour of the pattern during single twitches, we recorded the pattern during a time interval of 150 ms and in each run of 6–10 twitches this 'slot' was set at the same time during contraction. A total exposure time of 1–1.5 s was sufficient to record a good-quality pattern. The run was then repeated for a different time slice. Within this limited time resolution it could be seen that the disappearance of the layer-line pattern at the beginning of the twitch and its return during relaxation followed a time course very similar to tension development and its decay. This could be observed in quite short contraction series in which there was no doubt that the muscle was in good condition throughout and where there was none of the weakening of the resting layer-line reflections that we have observed in longer series of tetanic contractions, especially at lower temperatures; nor did we see the delayed return of off-meridional layer-line pattern after contraction apparent in very long contraction series¹³. Thus, the experiments showed unambiguously that the loss of the layer-line pattern is a specific and reversible accompaniment of normal contraction.

It was also noticeable, as observed previously⁴, that the decrease in intensity of the 14.3-nm meridional reflection was often much less marked than that of the off-meridional layer lines. Nevertheless, such muscles showed almost a complete disappearance of the 'forbidden' 21.5-nm meridional reflection during the peak of the twitch, and its reappearance during relaxation.

Intensity measurements with a one-dimensional detector

To compare the time course of the layer-line changes more accurately with that of tension, a different system was used. This used a position-sensitive one-dimensional proportional counter of the delay-line type, designed by Gabriel²². This was positioned over the muscle pattern with its axis parallel to the meridian but displaced sideways from it so as to record the strongest parts of the set of myosin layer-lines. When filled with a xenon-CO₂ mixture at 2 atm, the counter could record the first few layer-lines satisfactorily in a few seconds, giving a total of several thousand counts in the first layer-line after background stripping. (For stronger parts of the pattern, the beam had to be attenuated.) The delay line output was decoded using a direct time-digitising device (modified CERN modules DTD164 and 211)²³. The latter are read out by an auxiliary CAMAC crate controller designed by C. Boulin. This module stores the one-dimensional patterns in a memory linked to a PDP11/45 computer²⁴ in 10-ms time bins via a bin switching device designed and built by E. Dorrington. The time slots are synchronised electronically with the stimulation of the muscle. Details of this system will be described elsewhere. The X-13 camera²⁵ used in these later experiments is another mirror monochromator device again using an 8 × 20-cm section mirror but with a triangular-shaped monochromator which is bent into a section of circle by applying pressure at the apex²⁶. The specimen-to-detector distance was approximately 2.2 m.

With the storage ring operating in the dedicated mode at 3.185 GeV and 70–90 m/a (positrons only), with 480 bunches, total counting rates over one quadrant of the layer-line pattern were in excess of 10⁵ c.p.s. and adequate data could be collected in 10-ms time bins from a series of a few hundred single twitches spaced at 30-s intervals. Thus, a typical experimental run would last 2–3 h and gave a few seconds of total exposure time in each time bin.

In these experiments (and in the earlier ones) a fast-acting lead shutter was arranged so that exposure of the muscle to the X-ray beam was minimised, being kept to 2 or 3 min even in the longest runs. No evidence of radiation damage was apparent in these experiments. However, in test exposures exceeding 10–15 min, deterioration of both the X-ray diagram and the microscopic appearance of the region of the muscle in the X-ray beam was

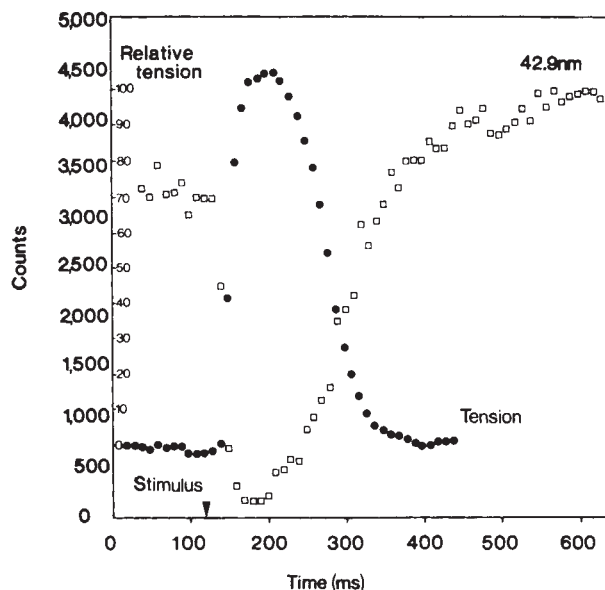


Fig. 2 Changes in the 42.9-nm layer line intensity (□) and the relative tension (●) during a twitch in frog sartorius muscle at 10 °C. Data are summed from 900 twitches, each time slot being of 10 ms duration, that is, corresponding to 9 s total exposure. Time at which electrical stimulus applied to muscle is shown by an arrow. Incident intensity at specimen was reduced by a factor of 3 (using aluminium foils) because of total counting rate limitations (later overcome) in the electronic circuits at the time of this experiment.

quite obvious. As such radiation effects have not apparently been observed with glycerinated insect flight muscle, it may be that the radiation-sensitive component resides in the calcium-releasing mechanism.

Data could also be collected from a much smaller number of twitches if longer time slots were utilised (for example, 50 ms), but no changes in the behaviour of the pattern were noted over the experimental times used. Meridional data were also collected, usually from considerable shorter series of twitches, as the patterns are much stronger and have a higher signal-to-noise ratio.

The layer-line reflections are superimposed on a steeply sloping background (see Fig. 1). A computer program was used to draw in the best fit (least squares) of a polynomial to the background levels seen between the successive layer-line peaks. It was found that a third (or sometimes fourth) order polynomial gave a satisfactory fit. If a separate fitting operation was carried out for each set of data in successive time slots, it was noticeable that a slight increase in the background level occurred during contraction. However, the most striking change was the large decrease in all the layer-line intensities during the time that the muscle was developing tension. The time courses of the changes in all the layer-lines do not seem to differ significantly and for simplicity we will consider only the first and strongest of these, that at 42.9 nm. The results of a typical run at 10 °C are shown in Fig. 2, where the intensity of the reflection is plotted as a function of time after the stimulus of the muscle (by a single maximal shock), in 10-ms time channels. The isometric tension developed by the muscle is shown on the same time scale. It can be seen that the reflection begins to decrease in intensity within 20 ms of stimulation, falls almost to zero intensity (in some cases) by about 70 ms, and then returns to its resting value (or even slightly more on several occasions) after about 200–250 ms.

The relationship between change in intensity and tension is shown more clearly in Fig. 3, where the change in each of them is plotted as a percentage of the maximum change occurring. It can be seen that both during the onset of activity and during relaxation, isometric tension and change in X-ray intensity follow a markedly similar time course; this was the case in all the records we obtained (six good runs so far) although in one or two

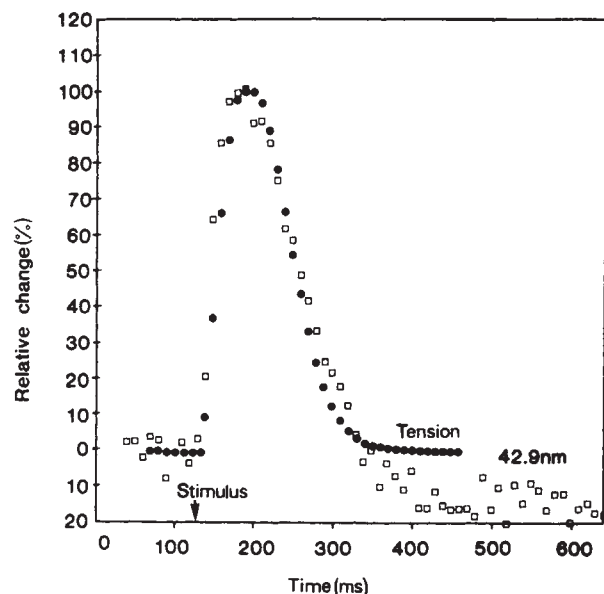


Fig. 3 Changes in the 42.9-nm layer line intensity ($\square$) and tension ($\bullet$) plotted as a percentage of maximal change. Other conditions are the same as in Fig. 2 except that the total exposure for each 10-ms time slot was only 3 s.

a very slight lag in the X-ray pattern (≤ 20 ms) was noted during relaxation. We do not have sufficient data, however, to establish whether or not the intensity change leads the tension change during the rising phase of tension by a few milliseconds at this temperature, as it does in the case of the equatorial reflections¹⁴.

Measurements of the intensity changes in the 14.3-nm meridional reflection in exactly the same conditions showed a rather interesting effect (Fig. 4). The change in intensity of the reflection, plotted again as a function of time after stimulus, exhibited a pronounced biphasic behaviour. The initial decrease in intensity took place with essentially the same time course as that of the layer-lines, but this was followed by a slight recovery of intensity during the peak of the twitch and then a large decrease in intensity again during the relaxation phase. Thus, the 14.3-nm intensity fell to its minimum value when the tension and layer-line intensities had almost returned to their resting values, and it returned to its resting value with a pronounced lag behind tension recovery. This lag has been seen in many previous experiments (using laboratory X-ray sources) and the biphasic fall in intensity has also been seen previously, although less frequently. This effect might be due to a longitudinal disordering of the myosin filaments, which in a resting muscle are arranged with their sets of cross-bridges in register. This would have a much larger effect on the intensity of meridional reflections than on the off-meridional layer-lines, and the disordering might be maximal during the relaxation phase, when different regions of a muscle relax at somewhat different rates and when disorder of the sarcomere pattern is much more frequent and pronounced than at other times during the twitch. We hope to investigate the phenomena further in future experiments.

Muscles at 'no-overlap' length

We have also carried out some preliminary experiments on the behaviour of the layer-line pattern in frog semitendinosus muscles stimulated at very stretched lengths where overlap of thin and thick filaments is either completely abolished or very greatly reduced. In earlier work using film and laboratory X-ray sources, in which very long tetani (7 s) had to be repeated many hundreds of times over a period of a day or more to record the patterns, a substantial decrease in layer-line intensity was observed, but it was not known whether this was due to an activation mechanism in the myosin filaments themselves, or whether it was associated with a progressive disordering of the

sarcomere structure allowing actin-myosin interaction still to occur at these extended sarcomere lengths^{7,9}. In the present experiments, using both the image-intensifier system and the position-sensitive counter system, we were able to record the layer-line pattern given by these stretched muscles by summing over as few as 10 1-s tetani, so that the possibility of the 'creep' artefact was very much reduced.

In all our experiments so far we have found a very large reduction in the extent of decrease of the layer-line intensities from very stretched muscles during stimulation compared with that seen in similar muscles at normal sarcomere lengths, and in most of the experiments no decrease at all in intensity was observed. For technical reasons it was not possible to determine sarcomere lengths during these experiments, in which active tension was reduced to about 10% of its rest-length value, but as the ability to develop full tension (and to show the normal layer-line changes) was restored when the muscles were returned to resting length, it seems likely that the muscles were close to the non-overlap length and in good condition. Thus, we conclude provisionally that interaction with actin is necessary for helical disordering of the myosin cross-bridges to occur following stimulation. This agrees with recent observations showing an absence of radial cross-bridge movement at no-overlap sarcomere lengths, as judged by the absence of change in the equatorial X-ray diagram¹⁶.

Interpretation of intensity changes

The principal finding we will discuss here is the large decrease in intensity of the layer-lines at resting sarcomere lengths. When this was first seen in the earlier experiments using film and long series of tetanic contraction^{4,5}, it was interpreted as indicating a disordering of the regular helical arrangement of myosin cross-bridges (in resting muscle), brought about by their interaction with actin during contraction. This interpretation remains a very plausible one, and is reinforced by the fact that the change is so closely correlated temporally with tension development, and that the bridges return to their normal helical order around each myosin filament immediately tension disappears (at least in relatively short series of twitches at 10 °C). During the onset of tension, although the ends of the muscles were held fixed, there would be significant internal shortening of the muscle against series elastic elements, equivalent to at least a 2 or 3% change in

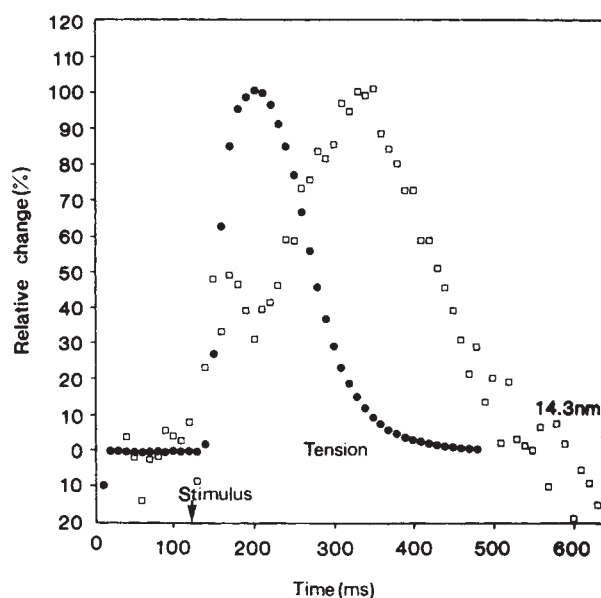


Fig. 4 Changes (plotted as a percentage of maximal changes) in the 14.3-nm meridional reflection ($\square$) and tension ($\bullet$) during a twitch from frog sartorius muscle at 10 °C. Total exposure is 1.5 s in each 10-ms time slot.

muscle length. This would correspond to several hundred angstroms of sliding movement between the overlapping actin and myosin filaments. Thus, if this movement was produced by a change in tilt of attached cross-bridges, we would expect to find the population of attached bridges distributed over all the possible angles of tilt, given the asynchronous nature of the interaction. This could explain why neither myosin type nor labelled actin type layer-lines are visible.

We consider, then, that these rapid and reversible changes in the myosin layer-line pattern during contraction provide further

good evidence that contractile force is produced by cross-bridges which attach to actin in the active muscle and undergo some form of tilting and pulling movement, as suggested previously²⁷. The experiments also demonstrate the usefulness of synchrotron radiation as a high-intensity X-ray source for time-resolved studies: it should also be possible to extend the scope of such studies considerably.

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Order and intracellular location of the events involved in the maturation of a spliced tRNA

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Microinjected frog oocytes were used to analyse the RNA processing steps which lead to the appearance of a mature cytoplasmic tRNA^{tyr} molecule. The results show that removal of the intervening sequence from within a yeast tRNA^{tyr} precursor, excision of extra 3' and 5' nucleotides, addition of a 3'-terminal CCA and modification of at least seven ribonucleotides all occur in the nucleus before the tRNA^{tyr} is transported to the cytoplasm. Moreover, we find that the ribonucleotide modifications occur in a strict order which precisely correlates with the size alterations of the tRNA^{tyr} precursor.

THE transcript of most eukaryotic genes is an RNA precursor which must be processed post-transcriptionally to yield a functional gene product¹⁻³. In the case of transfer RNAs the RNA processing or maturation includes size alterations and base modifications of an RNA precursor as well as transport of the RNA from the nucleus to the cytoplasm (reviewed by Smith in ref. 4). Furthermore, in the light of the discovery of intervening sequences in some tRNA precursors⁵⁻⁹, it is now clear that tRNA maturation sometimes involves the removal or splicing of intervening RNA sequences. At present, the intracellular location of the precursors and mature tRNAs and the relative order of the various processing steps is virtually unknown for eukaryotic tRNAs. This article describes how and where in the cell the RNA transcript of a yeast tyrosine tRNA gene is processed into a mature cytoplasmic tRNA.

The DNA sequences of four of the eight tyrosine tRNA genes (tDNA^{tyr}) of the yeast *Saccharomyces cerevisiae* have been determined (ref. 5 and P. Phillipsen, J. R. Cameron and R. W. Davis, personal communication). These data have shown that the tRNA^{tyr} genes contain a 14 base-pair intervening sequence, adjacent to the anticodon, which is not present in the mature tRNA. Previous studies on the expression of this gene in microinjected frog oocytes showed that the tDNA^{tyr} is first transcribed as a precursor RNA which has an extension at the 5' end of the mature molecule and an intervening sequence, but lacks a 3'-terminal CCA¹². In this article we analyse the processing of

the tRNA^{tyr} precursor in detail, focusing on the intracellular location of the RNA processing events. We conclude that (1) tRNA precursors with immature ends (having 5' leader and 3' trailer sequences) are located only in the nucleus, (2) removal or splicing of the intervening sequence occurs in the nucleus or at the nuclear membrane and (3) size alterations and base modifications occur in a strict temporal order.

Expression of cloned tRNA genes in injected oocytes

Amphibian oocytes have been used extensively as a biological test system for the expression of purified mRNAs and more recently for cloned genes (reviewed in ref. 13). It has been shown that cloned DNAs are accurately transcribed following injection into the nucleus of intact *Xenopus* oocytes¹⁴⁻¹⁸ or after addition *in vitro* to oocyte nuclear extracts¹⁹⁻²². Correct processing of the RNA transcripts in this system is demonstrated by the fact that oocytes injected with a cloned tRNA gene synthesise a tRNA precursor and process it into a mature tRNA having the correct size, sequence and even some of the appropriate base modifications^{12,18}. Moreover, the transcripts from the injected genes are translated into proteins in some cases, for example, SV40 DNA and sea urchin histone DNA^{23,24,46}.

In using this 'living test tube' to study the transcription of cloned DNAs many copies (usually about 10⁸) of one gene are injected into each oocyte. Consequently, the unusual situation exists in which most of the cells' newly synthesised RNA is

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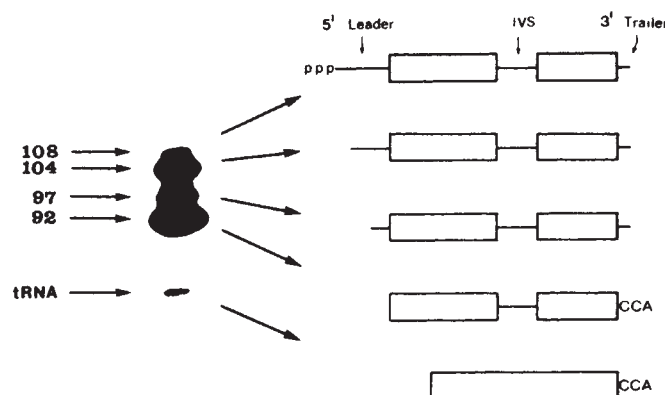


Fig. 1 Transcriptional products of $tDNA^{tyr}$ in injected oocytes. Oocytes were injected with $tDNA^{tyr}$ (plasmid pYTC (ref. 12), 5 ng per oocyte) and ^{32}P - α -GTP (10 mCi mol^{-1}), incubated for 6 h and the ^{32}P RNAs were extracted and electrophoresed in a 10% acrylamide gel as described¹⁸. The autoradiogram (left) shows the $tDNA^{tyr}$ precursors and mature $tRNA^{tyr}$ with their approximate lengths (± 4 bases). The structures of the precursors (right) were determined by comparing the RNA fingerprints (as in Fig. 4) with the DNA sequence as described in refs 5 and 12. The 5' leader is about 19 bases long, the intervening sequence is 14 bases long, the 3' trailer is 2 or 3 U, and the mature tRNA is 78 bases long. For the RNA sequences and a possible secondary structure of the precursors see ref. 12.

transcribed from just one type of gene: the injected template. When, for example, yeast $tDNA^{tyr}$ is injected into oocytes the transcripts from this gene, which include mature $tRNA^{tyr}$ and four $tRNA^{tyr}$ precursors, comprise up to 80% of the cells' newly synthesised RNA. This readily allows one to determine the structure of the tRNA precursors.

The intracellular location of the precursors and their processing enzymes can also be determined with this injection system²⁵. The *Xenopus* oocyte is such a large cell ($\sim 1.2 \text{ mm}$ diameter) that its nucleus or germinal vesicle ($\sim 0.4 \text{ mm}$ diameter) can be isolated by manual dissection. Thus, the newly synthesised RNAs present in the nucleus and cytoplasm can be analysed separately. Moreover, so many $tDNA$ transcripts are produced by one injected oocyte ($\sim 2,000 \text{ d.p.m. of } ^{32}P\text{-labelled RNA h}^{-1}$) that these transcriptional products can be analysed using a single injected oocyte.

tRNA Precursors with 5' leader and 3' trailer sequences do not leave the nucleus

When oocytes are injected with $tDNA^{tyr}$ and radioactive ribonucleotide triphosphates five main RNA species are synthesised. The structures of the $tRNA^{tyr}$ precursors, determined by RNA fingerprinting techniques, are shown schematically in Fig. 1. Note that the three longest precursors (108, 104, and 97 bases) all contain extensions at the 5' and 3' termini of the tRNA and an intervening sequence in the coding region. By a stepwise removal of the 5' leader sequence these precursors are processed into a 92-base precursor which has a mature 5' end, retains the intervening sequence, and has a 3' CCA. Finally, the 14 base-pair intervening sequence is removed giving rise to mature $tRNA^{tyr}$.

The intracellular location of the $tDNA^{tyr}$ transcripts was determined by dissecting oocytes which had been previously injected with $tDNA^{tyr}$ and $[\alpha\text{-}^{32}P]\text{GTP}$. In these experiments the RNAs present in the nucleus and in the cytoplasm were determined by analysing single oocytes. The results show a partitioning of the transcripts between the nucleus and cytoplasm (Fig. 2). All of the $tRNA^{tyr}$ precursors which contain 5' leader and 3' trailer sequences are found exclusively in the nucleus. The 92-base precursor which contains the intervening sequence, but has mature 5' and 3' termini, is found in both the nucleus and cytoplasm. The mature $tRNA^{tyr}$ (78 bases) is found predominantly, if not exclusively, in the cytoplasm.

After 2.5 h of $tDNA^{tyr}$ transcription most of the transcripts are still in the nucleus and little if any mature $tRNA^{tyr}$ has appeared (Fig. 2). By 6 h some mature $tRNA^{tyr}$ is detected in the cytoplasm and the 92-base precursor is the most prevalent transcript. By 24 h more mature $tRNA^{tyr}$ has accumulated in the cytoplasm, but the 92-base precursor is still the major transcriptional product.

From these results we conclude that tRNA precursors with extra tracts of RNA at their termini are localised in the nucleus. One trivial explanation for this observation is that the larger precursors are rapidly processed and/or degraded upon entering the cytoplasm. This possibility has been rendered improbable by experiments in which the 108-, 104- and 97-base precursors were injected directly into the oocyte cytoplasm and found to be stable. The nuclear segregation found for these $tRNA^{tyr}$ precursors has also been observed following microinjection of other tRNA genes, including nematode leucine

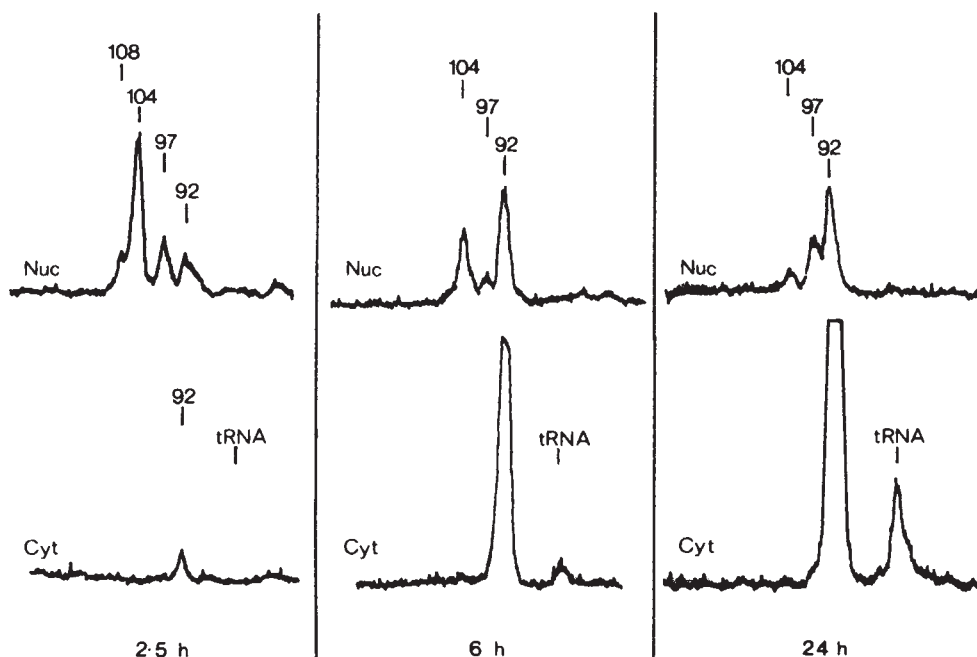


Fig. 2 Intracellular location of $tDNA^{tyr}$ transcripts. Single oocytes which had been previously injected with $tDNA^{tyr}$ and $[\alpha\text{-}^{32}P]\text{GTP}$ were manually dissected⁴² after incubation at 19°C for 2.5, 6 and 24 h and the RNAs present in each individual nucleus and cytoplasm were electrophoresed in 10% acrylamide gels¹⁸. The autoradiograms of these gels (like that in Fig. 1) were traced with a Joyce-Lobel densitometer. The tracings from three representative oocytes are shown. Nuc, nucleus; Cyt, cytoplasm.

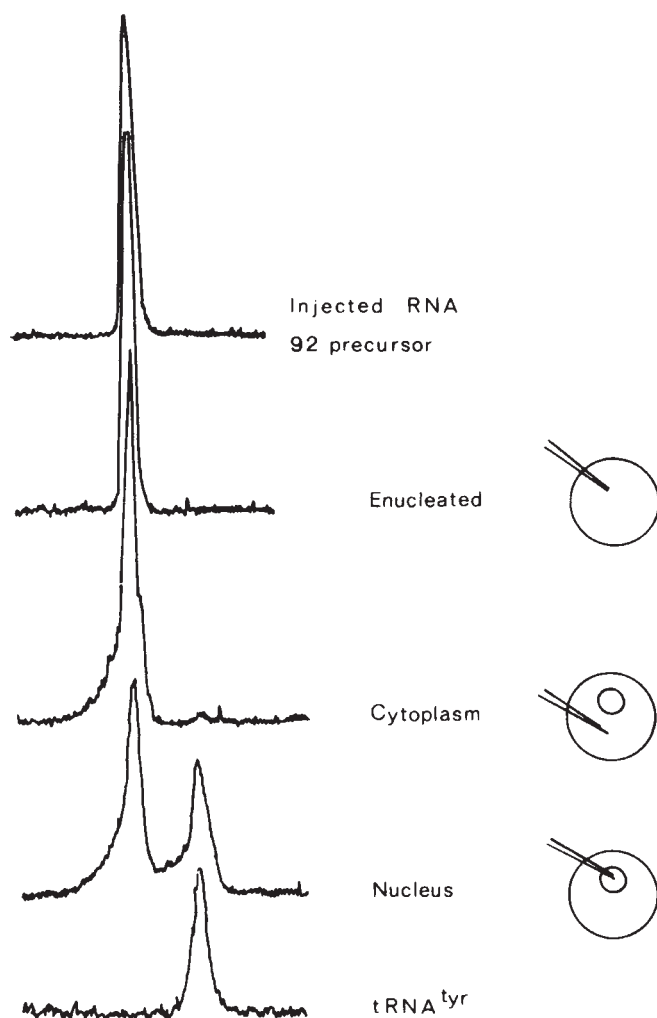


Fig. 3 Splicing of the intervening sequence is associated with the nucleus. Radioactive 92-base $tRNA^{tyr}$ precursor from injected oocytes was purified by extraction from acrylamide gels²⁵. This ^{32}P -labelled precursor, the substrate for the splicing enzyme(s), was injected into enucleated oocytes⁴³, the cytoplasm, or the nucleus of intact oocytes using approximately 5,000 d.p.m. of ^{32}P -labelled RNA per oocyte. After an 18-h incubation the injected oocytes were homogenised and the ^{32}P RNAs were extracted and electrophoresed in a 10% acrylamide gel. Two uninjected markers, for the 92-base precursor and the spliced $tRNA^{tyr}$, were electrophoresed in adjacent tracks. The densitometer tracings of this acrylamide gel are shown.

$tDNA^{25}$ and yeast serine $tDNA$ (J. Broach, R. Cortese and D. A. Melton, unpublished data). In all these cases, the common feature of the $tRNA$ precursors which are partitioned in the nucleus is that they have unfinished termini, that is, they have 5' leader and 3' trailer sequences.

Splicing of the intervening sequence occurs in the nucleus

A splicing activity has been isolated from nuclear preparations⁶ and from a cytoplasmic ribosomal wash in yeast⁸. However, the difficulties in obtaining pure preparations of nuclei and cytoplasmic fractions have led to uncertainties about the location of the splicing enzymes. Our results based on the dissection of injected oocytes (Fig. 2) show a similar ambiguity in that the 92-base precursor, the substrate for the splicing enzyme, is found in both the nucleus and cytoplasm whereas the mature $tRNA^{tyr}$ is found mostly in the cytoplasm. The two obvious possibilities for the removal of the intervening sequence are that

(1) the 92-base precursor is transported to the cytoplasm and then the intervening sequence is removed to give mature $tRNA^{tyr}$ or (2) the splicing occurs in the nucleus and the mature $tRNA^{tyr}$ is then quickly (or even as a consequence) transported to the cytoplasm.

We have tested the location of the splicing enzyme(s) in intact cells by injecting ^{32}P -labelled 92-base precursor RNA into the nucleus, the cytoplasm, and into enucleated oocytes. Figure 3 shows that a significant amount of the 92-base precursor is spliced only when the RNA is injected into the nucleus. This strongly suggests that the last step in tailoring the size of the precursor, the excision of the intervening sequence and subsequent ligation of the two $tRNA$ halves, is a nuclear event. Therefore, the splicing enzyme or enzyme complex might be in the nucleoplasm or located at the nuclear membrane. This conclusion is not affected by the problems posed by the biochemical purification of nuclei.

The splicing step is inefficient in injected oocytes: even after 6 d of $tDNA^{tyr}$ transcription only 30% of all the transcripts are spliced into mature $tRNA^{tyr}$ (ref. 12 and Fig. 2). The nuclear location of the splicing enzyme(s) could explain this inefficiency. Once the 92-base precursor enters the cytoplasm it is probably no longer accessible to the splicing enzyme(s). The 'leakage' of the 92-base precursor into the cytoplasm which we observe may result from the fact that the oocyte is overloaded with the transcripts of a single gene. One would not expect this to occur in normal yeast cells.

Post-transcriptional addition of CCA

All transfer RNAs have the sequence CCA at their 3' end, the attachment site for the amino acid. These three bases are not encoded in the yeast $tDNA^{tyr}$ and must therefore be added post-transcriptionally⁵.

The results presented in Fig. 4 show that the CCA is not present on the 104-base precursor. Analysis of the 97-base precursor (not shown) reveals that it has the same 3' terminus as the 104-base precursor, the CCA still being absent. By the 92-base precursor stage all of the molecules have a 3' CCA (Fig. 4f).

The presence of the CCA on the nuclear 92-base precursor suggests that these bases are added to the newly synthesised RNA in the nucleus. However, it seemed likely that the CCA-adding enzyme ($tRNA$ nucleotidyl transferase) would also be found in the cytoplasm because it is known that the 3' CCAs of $tRNAs$ are constantly removed and replaced or repaired in the cell^{26,27}. This was tested directly by injecting deadenylated $tRNA$ into different cell compartments. Deadenylated $tRNA$, a substrate for $tRNA$ nucleotidyl transferase, was prepared by chemically removing the 3'-terminal A residue from purified $tRNA^{tyr}$ (ref. 28). The results in Fig. 5 show that a 3'-terminal A residue can be added to deadenylated $tRNA^{tyr}$ in the cytoplasm as well as in the nucleus. Taken together with the results shown in Fig. 4, this shows that the enzyme $tRNA$ nucleotidyl transferase is present in both the nucleus and cytoplasm of the cell.

The experiments described so far suggest the following order for the size alterations of the $tRNA^{tyr}$ precursor: (1) stepwise removal of the 5' leader and 3' trailer sequences, (2) addition of a 3'-terminal CCA and (3) splicing of the intervening sequence. All of these processing events occur in the cell nucleus. In addition to these processing steps, the maturation of a $tRNA$ molecule involves modification of specific ribonucleotides. The relationship between the size alterations and base modifications of the $tRNA^{tyr}$ precursors is described below.

Ribonucleotide modifications occur in an obligatory order

The intracellular site at which the ribonucleotides are modified was examined by analysing the RNA fingerprints of the nuclear and cytoplasmic $tRNA^{tyr}$ precursors. The T_1 ribonuclease fingerprints of the nuclear 104-base, nuclear 92-base and cytoplasmic 92-base precursors are shown in Fig. 4. The minor bases

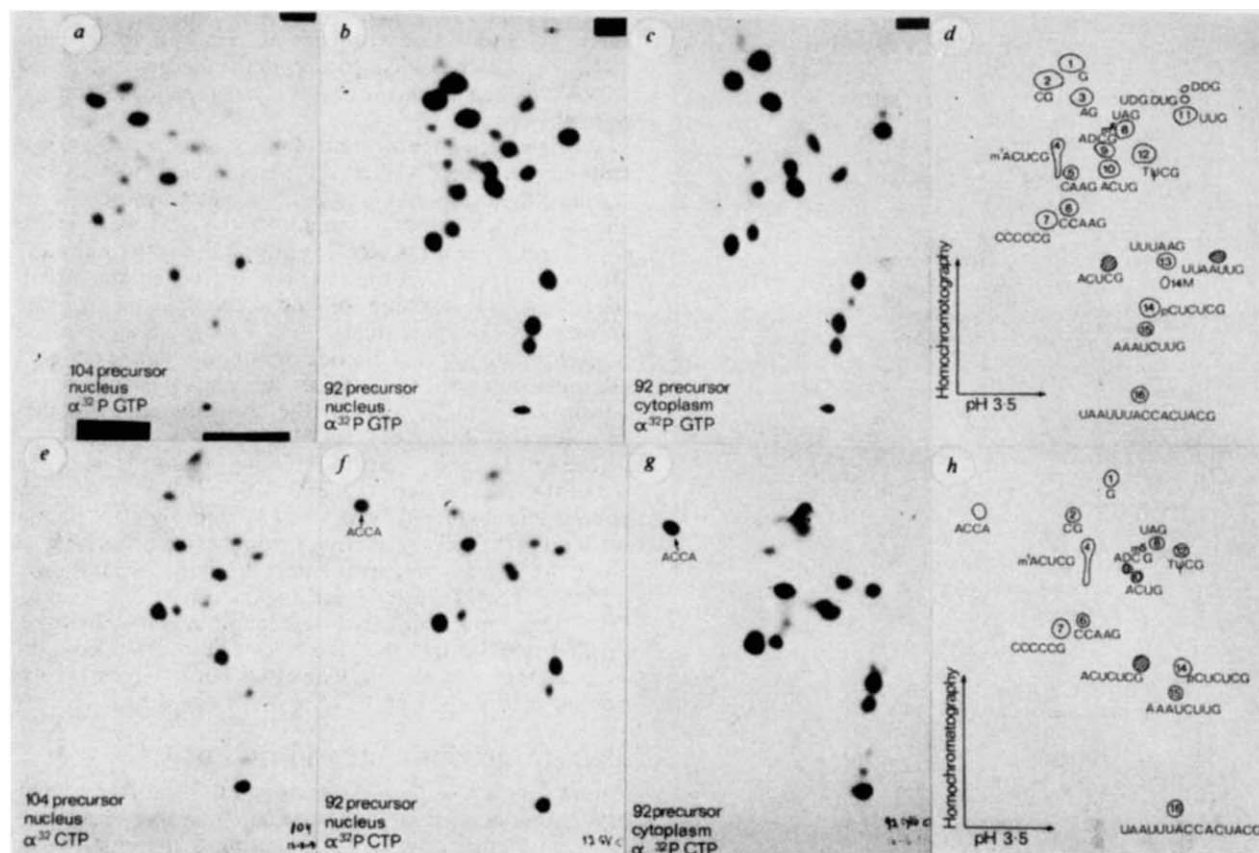


Fig. 4 RNA fingerprints of tRNA^{tyr} precursors synthesised in injected oocytes. ³²P precursor RNAs were isolated either from the nucleus or the cytoplasm of oocytes 5 h after injection with tDNA^{tyr} and [α-³²P]GTP (a–d) or [α-³²P]CTP (e–h). Following digestion with ribonuclease T₁ the oligonucleotides were separated in one dimension on cellulose–acetate at pH 3.5 and in the second dimension by homochromatography on DEAE–cellulose thin layer plates⁴⁴. Some background of small oligonucleotides from endogenous oocyte transcripts is apparent in the upper part of panels a and b. This is because the RNA bands analysed were relatively weak and the fingerprints had to be exposed for long periods. The diagrams at the right indicate the composition of the oligonucleotides; hatched spots are oligonucleotides unique to the 104-base precursor. For the numbering of the T₁ oligonucleotides and their position within the tRNA^{tyr} precursor see refs 5 and 12. Note the 3'-terminal CCA is present in the 92-base precursors (f, g), but absent in the 104-base precursor (e). Some base modifications affect the mobility of certain oligonucleotides, for example D in oligonucleotide 9, as discussed in the text.

present in the precursors were analysed by elution of the T₁ oligonucleotides, hydrolysis with alkali and thin layer chromatography²⁹. Table 1 summarises the results of this type of nearest neighbour analysis for some selected T₁ oligonucleotides, and shows that most of the base modifications analysed, including 5-methyl-cytosine (m⁵C), 1-methyladenosine (m¹A), pseudouridine (Ψ), and dihydrouridine (D), occur in the nucleus. The only modification found to be exclusively cytoplasmic is a G modification in T₁ oligonucleotide 14. This uncharacterised modification (see Table 1) causes the appearance of a new spot in the RNA fingerprint of the cytoplasmic 92-base precursor (see Fig. 4c, oligonucleotide 14M). It should be noted that although this nucleotide is not normally modified in yeast, all the other modifications described in Table 1 are normally present in yeast tRNA^{tyr}.

Analysis of the RNA fingerprints of the various tRNA^{tyr} precursors (as in Fig. 4) also showed that base modifications occur in an obligatory order which is strictly correlated with the size alterations of the tRNA precursors. Some modifications are present on precursors which have 5' leader and 3' trailer sequences while others do not occur until these sequences are removed (Table 1).

The correlation between the size reduction and base modification of the precursors is easily demonstrated in the cases where the base modifications cause a change in the mobility of a T₁ oligonucleotide. For example, oligonucleotide 9, which has the sequence ADm⁵CG, can be resolved from oligonucleotide 10, ACUG, only when the dihydrouridine (D) is present. In the 104-base precursor the two oligonucleotides, 9 and 10, overlap

(Fig. 4a) whereas in the 92-base precursor, which contains D in oligonucleotide 9, the two spots are separated in the fingerprint (compare Fig. 4a with 4b and c). This particular modification, U→D, is observed only in precursors which have had the 5' leader removed and the 3' CCA added. Other modifications, as indicated in Table 1, are found on precursors containing 5' leader and 3' trailer sequences. In the case of oligonucleotide 4, m¹ACUCG, the methylation of the adenosine causes an increased mobility in the second dimension of the fingerprint analysis. This nucleotide is already completely modified to m¹A at the 104-base precursor stage (Fig. 4e).

Perhaps the most striking example of the order in tRNA processing is provided by the case of two pseudouridine (Ψ) modifications. In the 104-base precursor there is a Ψ in the TΨC loop (oligonucleotide 12), but the Ψ which will eventually appear in the anticodon stem (oligonucleotide 15) is absent. Once the 5' leader sequence is removed and the 3' CCA added, the U in the anticodon stem of the 92-base precursor is completely modified to Ψ (Table 1). It should be noted that only a selected subset of the possible base modifications was analysed here. Other base modifications may well occur.

We therefore observe an apparently invariant order in the processing of the tRNA^{tyr} precursors. The order of events is said to be invariant in that we do not find, for example, precursors which have had the intervening sequence removed but still retain a 5' leader sequence. Nor, for example, are certain nucleotides modified in precursors which still have a 5' leader. Conventionally, the maturation of tRNA precursors is divided into two separate processes: size reduction and base

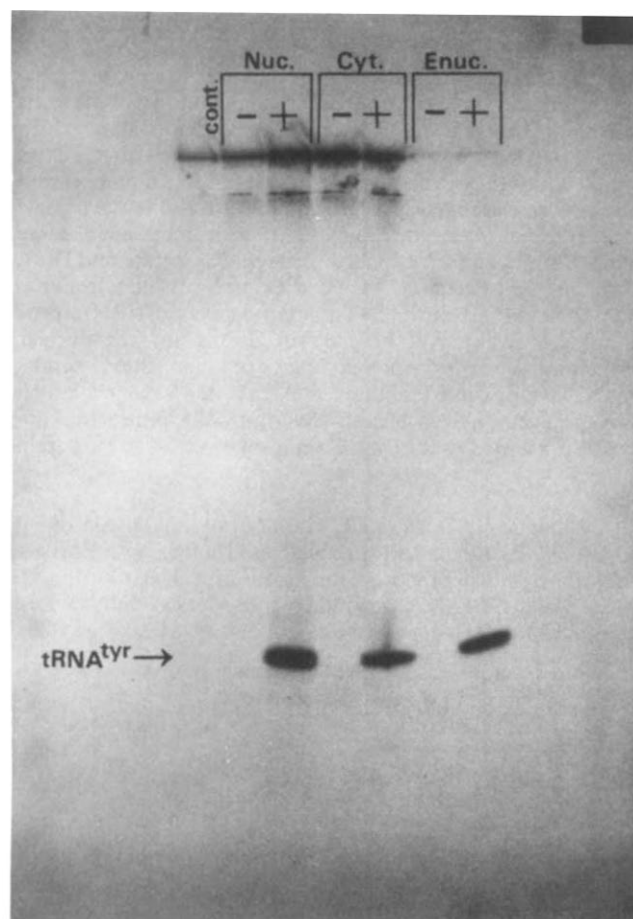


Fig. 5 Addition of a 3'-terminal A residue to transfer RNA in injected oocytes. [α - 32 P]ATP (10 mCi ml $^{-1}$) was injected with (+) or without (–) deadenylated tRNA^{tyr} (25 ng per oocyte) into either the nucleus (Nuc) or the cytoplasm (Cyt) of intact oocytes and into enucleated oocytes (Enuc.). After an incubation of 1.5 h the RNA was extracted and electrophoresed in a 10% acrylamide gel as in Fig. 1. Pure tRNA^{tyr} (provided by M. Olson) had the 3'-terminal A removed by periodate treatment²⁸. Pure tRNA^{tyr} which had not been treated with periodate was injected into oocytes with [α - 32 P]ATP as the control (cont.).

modification. The results presented here show that these processes are in fact intimately related and proceed in concert with each other.

Discussion

A plausible scheme for the steps involved in the biosynthesis of yeast tRNA^{tyr} as observed in injected oocytes is shown in Fig. 6. Perhaps the most striking aspect of this pathway is that almost all of the RNA processing occurs in the nucleus. From the nuclear location of the tRNA^{tyr} precursors one can infer that certain maturation enzymes must be located in the nucleus. These include the enzymes that synthesise 1-methyl-adenosine, 5-methyl-cytosine, pseudouridine, and dihydrouridine (Table 1). Furthermore, experimental evidence has been presented to show that the removal of the 5' leader sequence, splicing, and addition of the 3' CCA all occur in the nucleus (Figs 3, 5, ref. 25). Earlier studies based on the biochemical fractionation of nuclei and cytoplasm indicated that tRNA precursors are found and processed in the cytoplasm^{26,30–33}. With different methods, namely the manual isolation of the oocyte's large nucleus and the direct injection of radioactive RNAs into various cell compartments, the results reported here and those of Lönn³⁴ support the conclusion that almost all of the maturation of tRNA precursors occurs in the nucleus.

Table 1 Order and intracellular location of base modifications

Ribonucleotide modification	T ₁ oligonucleotide	Precursor (bases)		
		104 nucleus	92 nucleus	92 cytoplasm
m ¹ A	4 (TΨC loop)	+	+	+
m ⁵ C	9 (variable loop)	+	+	+
Ψ	12 (TΨC loop)	+	+	+
	15 (anticodon stem)	–	+	+
D	9 (variable loop)	–	+	+
	11 (D loop)	–	+	+
G*	14M (aa stem)	–	–	+

The 104-base and 92-base tRNA^{tyr} precursors were isolated from either the nucleus or cytoplasm of oocytes which had been injected with tDNA^{tyr} and [α - 32 P]GTP or [α - 32 P]CTP. The modified bases present in selected T₁ oligonucleotides were characterised by pancreatic RNase digestion or alkali hydrolysis followed by cellulose thin layer chromatography in isopropanol: H₂O:HCl (70:15:15 v/v)⁴⁵ or paper electrophoresis at pH 3.5 (ref. 44). The T₁ oligonucleotides are numbered as in Fig. 4. Note that most of the modifications occur in the nucleus (except for G*) and that some modifications only occur after the termini of the precursor have been matured. m¹A, 1-methyladenosine; m⁵C, 5-methylcytosine; Ψ, pseudouridine; D, dihydrouridine; G* is an uncharacterised G modification whose migration by paper electrophoresis is consistent with it being Gm (2-O-methylguanosine) or some other modification that renders G resistant to ribonuclease T₁ cleavage.

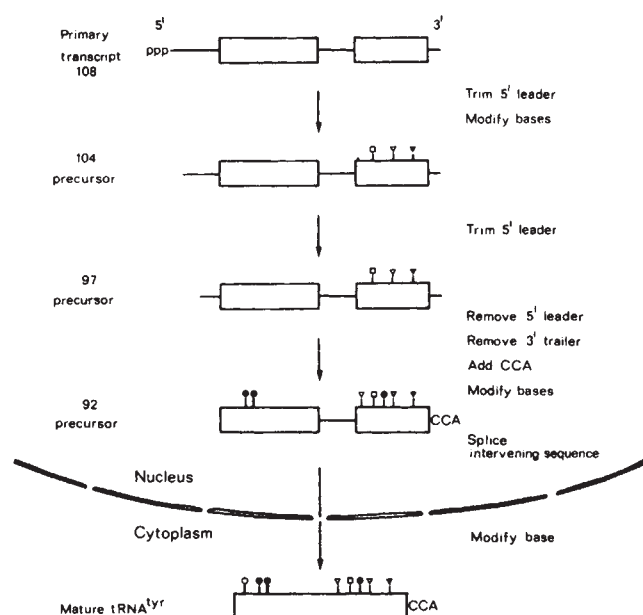


Fig. 6 RNA processing of tRNA^{tyr} precursors into mature tRNA^{tyr} in injected oocytes. The primary transcript of tDNA^{tyr} is a precursor 108(±4) nucleotides in length with a 5' leader, intervening sequence and 3' trailer (as in Fig. 1). This is the only precursor which has a 5'-terminal triphosphate¹². The 5' leader is removed in three stages, the last of which is accompanied by excision of the 3' trailer and addition of the 3' CCA. The position and type of the base modifications are indicated as well as the stage at which they appear. The modified bases in the 108-base and 97-base precursors have not been analysed. Therefore some of the modified bases shown to be newly added at the 92-base precursor stage may be present on the 97-base precursor, but these are definitely absent in the 104-base precursor (Table 1). Note that only some base modifications have been analysed; others may well occur. The 92-base precursor which enters the cytoplasm (Fig. 2) is not shown because our results suggest that the cytoplasmic 92-base precursor is not processed to mature tRNA (see text). □, 5-methyl-cytosine; ▽, pseudouridine; ▼, 1-methyladenosine; ●, dihydrouridine; ○, G*, an uncharacterised G modification.

Superimposed on the spatial order of the tRNA processing we have observed a correlation between the size changes and base modifications of the tRNA precursors. Previous studies have suggested that the length reduction of tRNA precursors occurs in a precise order^{4,35-39}, but little work has been done on its connection to base modification. Two notable exceptions from studies on bacterial tRNAs are the demonstration that a Gm modification is absent in a bacteriophage T₄ tRNA precursor⁴⁰ and *in vitro* studies on the effect of pseudouridine modifications on *E. coli* tRNA^{tr} cleavage by RNase P (ref. 41). The obligatory order observed for the yeast tRNA^{tr} base modifications can be explained by assuming that each of the tRNA^{tr} precursors is a substrate for a particular subset of maturation enzymes. For example, the presence of a 5' leader sequence could introduce order into the maturation process by altering the tertiary structure of the precursor. Some maturation enzymes might recognise precursors with a 5' leader while others would accept the precursor as a substrate only after the 5' leader has been removed. The specific sequence in which the bases of the 104-base precursor and then the 92-base precursor are modified provides some support for this suggestion (Fig. 6). The possibility remains that the intervening sequence might similarly order an additional round of base modifications, but this was not tested in the present study.

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In conclusion, it is not immediately obvious why a gene product as apparently simple as a tRNA is synthesised via such a complicated processing scheme. One hint, however, comes from our previous observation¹² that different genetic loci of the same tRNA gene are transcribed into RNA precursors all of which have a 5' leader, though the length and sequence of this extra segment varies in each case. The fact that the 5' leader is present in the case of the four yeast tyrosine tDNAs and the two yeast serine tDNAs examined suggests that it may have some biological function (ref. 12 and J. Broach, R. Cortese and D. A. Melton, unpublished data). Our studies implicate the 5' leader as having a possible role in two related aspects of tRNA processing: the ordering of base modifications and the nuclear segregation of tRNA precursors. Together these results suggest that a possible function for extra terminal sequences on tRNA precursors is to ensure that normally only a mature tRNA is presented to the cytoplasm for participation in protein synthesis.

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LETTERS

IUE observations of the hot components in two symbiotic stars

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Recent IUE observations reveal striking differences in the UV spectra of two symbiotic stars, R Aqr and RW Hya. RW Hya is found to be an unexpectedly intense source of UV radiation. The measurements reported here demonstrate the presence of a hot component in each star, supporting the view that each is a binary system with a luminous red primary and a hot, sub-luminous companion. In one case, the hot companion manifests itself by exciting a compact nebulosity; in the other case we believe that the continuous spectrum of the hot star is directly detected, while the continuum of nebulosity excited by the hot star is detected at longer wavelengths.

Our observations were made in the wavelength range 1,200-3,200 Å. The UV continuum found in the spectrum of R Aqr and shown in Fig. 1a is most easily interpreted as emission from a dense and compact nebula, which must be ionised by UV photons from a hot secondary. From visible light spectra, R Aqr is classified¹ as M7+pec. The continuum seen in Fig. 1a is ascribed to Balmer emission that arises from recombination radiation of hydrogen atoms. The nebula also produces both permitted and semi-forbidden lines of various ions. Observations in the IUE long-wavelength range (1,900-3,200 Å) also show the forbidden line of O II at λ 2,470 Å and a strong emission feature at λ 2,328 Å that must represent a blend of λ 2,321 Å [O III] and $\lambda\lambda$ 2,325, 2,327, 2,328 Å C II]. The most prominent emission lines include C III], C IV, Si II, Si III] and Si IV (Fig. 1a). Lower intensity features are not identified since blends due to the ~6 Å spectral resolution make some identifications of weak lines ambiguous. The $\lambda\lambda$ 1,808 and 1,806 Å lines of Si II, for example, most likely correspond to the broad emission feature evident in Fig. 1a.

The relatively low excitation spectrum of the nebula indicates that the nebula is excited by a star (with effective temperature $T \geq 50,000$ K) that resembles a white dwarf or the central star of a planetary nebula. Only such a star can produce enough

ionising photons as required by the present measurements ($\sim 10^{42} \text{ s}^{-1}$) and yet be sufficiently faint (given the estimated distance of $\sim 260 \text{ pc}$) (ref. 1) so that the stellar continuum does not dominate the UV spectrum of R Aqr. Our computations are based on an estimated colour excess, $E(B-V) \approx 0.05 \text{ mag}$, corresponding to an interstellar extinction at wavelength $1,550 \text{ \AA}$, $A_\lambda \approx 0.37 \text{ mag}$.

The feature at $\sim 1,250 \text{ \AA}$ is indicated in Fig. 1 as composite emission of S II and N V. However, presumably it is dominated by S II as the relatively low excitation of this ion is consistent with the nebular temperatures deduced from the oxygen and carbon line intensities. Our low spectral resolution observations cannot positively differentiate one ion species from another in this feature. However, we find that a compact dense nebula of $n_e \sim 10^6 \text{ cm}^{-3}$ and temperature $T \geq 15,000 \text{ K}$ suggests that it is due mainly to S II rather than the higher excitation emission line of N V. Also, the UV emission observed is not adequately explained by a hot chromosphere in the M giant primary, because the densities required to produce other emission lines observed ($n_e \sim 10^7\text{--}10^8 \text{ cm}^{-3}$) at typical chromospheric temperatures $T \leq 10,000 \text{ K}$ at these densities would be inconsistent with the presence of [O III] emission at $2,321 \text{ \AA}$ that is observed in the IUE long-wavelength range.

RW Hya is classified gM2 + pec and varies between 10 and 11 visual magnitudes. Attributing the visual flux to the red giant primary, we estimate the distance to the system as $\sim 1 \text{ kpc}$. Although the presence of hot companions in symbiotic stars has been suspected, the intensity of UV radiation from RW Hya is surprising. The resonance lines of the C IV ion at wavelengths $1,548, 1,550 \text{ \AA}$ are so bright that they saturate in a single pixel in an exposure time of 30 s with the short wavelength, low dispersion IUE spectrometer. The star appears to be essentially unreddened and is notable for the strong UV continuum that (Fig. 1b) rises towards short wavelengths. The distance of $\sim 1 \text{ kpc}$ deduced for RW Hya was obtained from the interstellar absorption of Ly α that produces an absorption equivalent width $w_{\lambda 1,216} \sim 1.5 \text{ \AA}$. The corresponding hydrogen column density in our line of sight $N_{\text{H I}} \sim 6.4 \times 10^{18} \text{ cm}^{-2}$ corresponds to an extinction $E(B-V) \approx 0.001$. This small absorption value is attributed to the height of RW Hya above the galactic plane ($l = 316^\circ$, $b = +36.5^\circ$) which places the star at a distance $\sim 700 \text{ pc}$ above the plane of the galaxy.

The continuum observed in RW Hya fits a Rayleigh-Jeans approximation to a black-body law that corresponds to an effective temperature $T \geq 10^5 \text{ K}$, with a peak that probably occurs well below the wavelength range of IUE near $500 \text{ \AA}$. Model atmospheres for hot white dwarfs show that in the UV range over which we observe a strong continuum, the thermal emission of such stars is approximated closely by that of a black body⁵. This must represent the emission of a subluminescent star, brighter and hotter than the companion of R Aqr. The continuum of the hot component in RW Hya, if it continues to follow the Rayleigh-Jeans law, must fall well below the continuum that we actually observe above $1,900 \text{ \AA}$ with the long wavelength, low dispersion IUE spectrograph. The long wavelength UV continuum, much too bright to arise in the M primary, is ascribed to nebulosity excited by the secondary star. Although both permitted and semi-forbidden emission lines are found, we have not identified any forbidden line in the UV spectrum of RW Hya, suggesting that the nebulosity in this star may be more dense (and perhaps smaller) than that in the R Aqr system.

The physical conditions that are responsible for the UV emission from these two symbiotic stars are notably different. The relatively low excitation emission observed in the line spectrum of R Aqr is indicative of a nebula photoionised by the hot star in the system. The prevailing density in the nebula, as deduced from the carbon and forbidden oxygen line strengths, must be roughly 10^6 cm^{-3} , while the scale of the nebula is $\leq 10^{15} \text{ cm}$ and the electron temperature is $\approx 15,000 \text{ K}$. The electron density and temperature of the nebula in which the primary and secondary are embedded was obtained from the

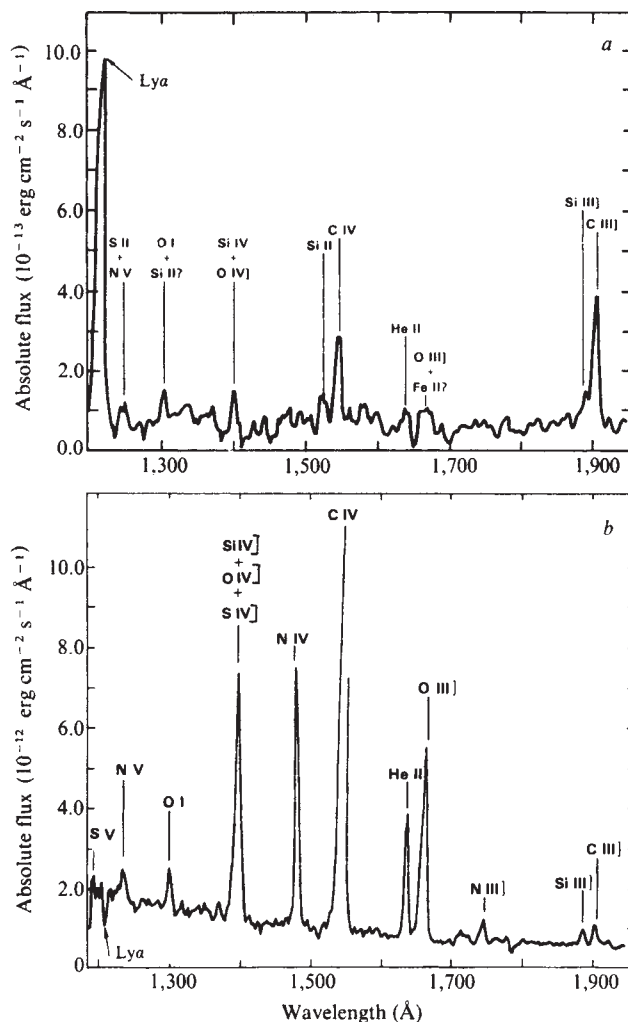


Fig. 1 UV spectra of the symbiotic stars R Aqr (a) and RW Hya (b) obtained with the short wavelength, low dispersion spectrograph of the IUE observatory satellite on 29 July 1979 and 1 September 1979 respectively. Note in b that the resonance lines of C IV at $1,548 \text{ \AA}$ and $1,550 \text{ \AA}$ are off scale and that the pronounced continuum rises towards shorter wavelengths, and that Ly α is in absorption. The spectra have been corrected for the absolute response of the instrument, but not for interstellar reddening, which affects the R Aqr spectrum. The Ly α emission line observed in R Aqr represents combined geocoronal and stellar emission.

relation and parameters of Osterbrock² for planetary nebulae, where we have used appropriate cosmic abundances for oxygen and carbon. Here the observed flux for a particular emission line is assumed to be proportional to $n_e^2 L^3$ for a given temperature, distance and cosmic abundance. Note that the scale size L of the nebula is comparable to the diameter of the hot star's orbit, if the orbital period is $\sim 27 \text{ yr}$ (ref. 3).

In contrast, the lack of significant emission in the $2,321 \text{ \AA}$ line of [O III], and the strong permitted line emissions, suggest that the nebulosity in RW Hya is much denser, $\sim 10^9 \text{ cm}^{-3}$, and hotter, $T_e = 20,000 \text{ K}$. The strong permitted line emissions, such as those due to C IV and O I, may be the result of mechanical heating of the gas as the result of tidal interaction between the extended envelope of the red giant primary and the compact companion. Mass accretion onto the surface of the companion can result in thermal heating of the accreting surface⁴. The He II line at $1,640 \text{ \AA}$ observed in both symbiotic stars is observed to be far more intense in RW Hya than R Aqr, confirming that RW Hya is a higher excitation source. The relatively weaker He II $1,640 \text{ \AA}$ emission observed in the spectrum of R Aqr probably reflects the lower ionisation of a nebula that is excited by a relatively cooler companion star. The presence of this high temperature line is possibly explained by emission arising in very

close proximity to the hot component with coronal temperatures.

Mass transfer may be a significant energy source in RW Hya and may be related to the intense UV emission and to the higher apparent effective temperature of its companion star, as compared with R Aqr. As in other types of binary systems, a distinguishing characteristic among individual symbiotic stars may be the degree of mass transfer onto the compact star. This is supported by IUE observations of CH Cyg, which reveal an UV continuum ascribed to mass transfer from the M6-M7 III primary on to the hot subluminescent secondary which results in an optically thick UV-emitting corona around the secondary⁶, a model supported by optical spectra as well. Given the energetics of the accretion process in white dwarfs, luminosities of $\sim 10^{36}$ erg s⁻¹ in the X-ray regime (1-4 keV) seem possible⁴. Accordingly, RW Hya may be a detectable source with the imaging proportional counter on the HEAO 2 (Einstein) observatory.

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A model for ball lightning

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An eyewitness sees ball lightning as a luminous sphere in regions of thunderstorm activity. There are many reports of ball lightning forming after a lightning discharge, existing for several seconds, and terminating suddenly and silently, or exploding with or without material damage^{1,2}. Jennison³ has described ball lightning observed at close range inside a commercial aircraft shortly after a lightning discharge. As it drifted down the aisle of the passenger cabin, this ball lightning exhibited: (1) perfect equilibrium in a perfectly spherical shape with diameter 22 ± 2 cm; (2) 5-10 W of optical radiation in blue-white light, but no heat radiation; and (3) an optically thick surface of almost solid appearance, devoid of polar or toroidal structure, but with some limb-darkening. It seems unreasonable to dismiss Jennison's ball lightning as an optical illusion⁴ (afterimage of the eye's retina). A physical explanation in terms of a microwave cavity⁵, a d.c. glow discharge⁶, chemical reactions⁷, nuclear reactions⁸ or annihilation of antimatter⁹ runs into difficulties since the all-metal airplane structure would act effectively as an air-tight Faraday cage. A plethora of excited atoms and molecules, and electromagnetic waves ranging from radio frequencies to X rays are produced in the channel of a lightning discharge, but only electric charge itself can enter the interior of an all-metal airplane without difficulty. Here a new model is presented which explains ball lightning in terms of electric charge.

Charge transfer through ionised air generally obeys Ohm's law $\mathbf{j} = \sigma \mathbf{E}$, where $\mathbf{j}$ stands for current density and $\mathbf{E}$ for electric field. The scalar factor σ is the electrical conductivity. The discharge current magnetises the ionised region where it passes. This magnetisation is dissipated into heat on a time scale known as the magnetic diffusion time τ . Magnetic field lines are 'frozen into' the plasma for a time span¹⁰:

$$\tau \approx \mu \sigma L^2 \quad (1)$$

where μ is magnetic permeability, σ is electrical conductivity and L is a typical length scale of the ionised region. From his measurements of the emission line profile of H α , Uman¹¹ has calculated a conductivity of 1.8×10^{-4} S m⁻¹ during the high-temperature phase of a lightning discharge. With vacuum permeability $\mu_0 = 4\pi \times 10^{-7}$ N/A², and with 10 cm diameter for the lightning channel, equation (1) yields a magnetic diffusion time of 2.3×10^{-4} s. This short time scale is clearly incompatible with observed ball lightning lifetimes, but it can easily account for instantaneous disappearance of a magnetised plasma sphere in air.

Equation (1) offers two ways of suppressing magnetic dissipation by ohmic losses in the plasma. First, the magnetic permeability μ could be larger than the vacuum permeability μ_0 , as in ferromagnetic materials. Below the Curie temperature, a ferromagnet has a permanent magnetic field, corresponding to $\mu = \infty$ in equation (1). Second, the electrical conductivity σ could be higher than in the lightning channel. Infinite conductivity is found in superconducting materials below the transition temperature, where electrical currents persist indefinitely. Uman's lightning channel conductivity of 1.8×10^{-4} S m⁻¹ holds good for laminar plasma motion only. Plasma turbulence will enhance charge transfer by translations of charged fluid parcels, and rotations of charged fluid parcels will generate a magnetic field. Our first hypothesis is that turbulence in the ball lightning plasma enhances charge transfer to the point that charged vortices transport electricity the way electrons do in a superconductor. By this hypothesis, individual plasma vortices have a strong tendency to travel with the same momentum and spin with aligned axes; as such they behave macroscopically the way bosons do on the atomic level.

Feynman¹² has shown that a fluid consisting of charged bosons has potential energy of the form $\nabla^2 \rho^{1/2} / \rho^{1/2}$, where ρ stands for the charge density, or, by virtue of Coulomb forces between electrons and ions, for mass density. This potential energy is zero for a uniform distribution ρ is constant (as in solids), but it can be positive as well as negative in a density-stratified plasma with non-uniform ρ . Correcting the sign of Feynman's expression we write $U = -C \nabla^2 \rho^{1/2} / \rho^{1/2}$ ($C > 0$) for the potential energy U , corresponding to interaction between plasma vortices with a force field $-\nabla U = \nabla C \nabla^2 \rho^{1/2} / \rho^{1/2}$. In hydrostatic equilibrium this force field is balanced by pressure forces only, hence¹⁶:

$$0 = \frac{-1}{\rho} \nabla p + \nabla C \frac{\nabla^2 \rho^{1/2}}{\rho^{1/2}} \quad (2)$$

where the total pressure p includes kinetic pressure from electrons and ions, and turbulent pressure from plasma vortices.

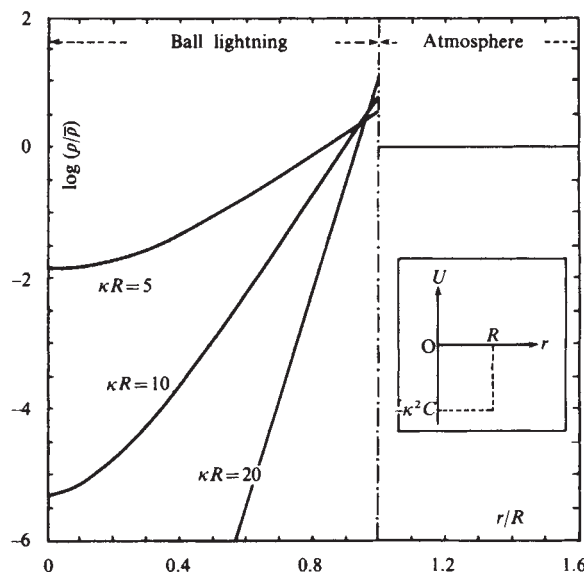


Fig. 1 Three density profiles of isobaric ball lightning models with different binding energies. Inset, square well potential energy of isobaric density profiles.

Each plasma vortex exerts centrifugal forces on its neighbours, which dominate over kinetic pressure when plasma circulation inside the vortex exceeds the local speed of sound. Such supersonic circulation causes density gradients on the length scale of one plasma vortex, resulting in a sponge-like structure of tenuous vortex cores nested in dense ambient plasma.

Collective motions of electrons and ions on length scales smaller than one Debye length are dissipated by Landau damping¹⁵, and local deviations from charge neutrality are screened out on length scales larger than one Debye length¹⁵. Thus the sizes of plasma vortices behaving as charged bosons apparently lies around one Debye length. Since Debye length scales as $T^{1/2}/n^{1/2}$ with temperature T and electron density n , a hot and tenuous region of the ball lightning plasma has larger vortices with more electrons and ions than a cooler and more dense region. High-pressure arc plasmas typically have a Debye length around 10^{-6} cm, which may serve as a lower limit on the size of plasma vortices in our ball lightning model.

The virial theorem⁶ yields $3pV$ as an upper limit on the energy content of a plasmoid with pressure p and volume V . Confinement by atmospheric pressure only, results in a maximum energy content of $\sim 10^3$ J, which is several orders of magnitude below ball lightning energies reported elsewhere^{1,8}. Some ball lightning theories avoid this difficulty by postulating a continuous energy input through radiowaves⁵ or d.c. currents⁶. Our boson model for plasma turbulence lifts the virial constraint on maximum energy content with negative potential energy from interaction between plasma vortices. With internal confinement forces as in equation (2), hydrostatic equilibrium of the ball lightning plasma demands internal pressures above 1 atm. The energy $\int p dV$ stored in the pressure distribution includes thermal energy from electrons and ions, and turbulent energy from vortex motion.

Equation (2) is nonlinear except in the isobaric case where p is constant. For spherical symmetry, the non-singular isobaric solution with negative potential energy $U = -\kappa^2 C$ is^{13,16}:

$$\rho^{1/2} = \rho_C^{1/2} \frac{\sinh(\kappa r)}{\kappa r} \quad (3)$$

where ρ_C denotes the central density of the plasma sphere, and $1/\kappa$ is a length scale. In the limit $\kappa \rightarrow 0$, equation (3) represents a uniform and isothermal plasma sphere without potential energy. Density stratification appears as κ increases from zero, giving progressively more negative potential energies $-\kappa^2 C$, and lower central densities ρ_C . A complementary stratification in temperature maintains isobaric conditions in the gas. Thus equation (3) represents a plasma sphere with a hot and tenuous central region surrounded by a cooler and more dense 'gas blanket'.

For a ball lightning floating in air, the mean density $\bar{\rho}$ cannot be far from atmospheric density, 1.3 kg m^{-3} . On squaring and integrating equation (3) over the volume of a sphere with radius R , the mean density comes out as $\bar{\rho} = 3\rho_C \{ \sinh(\kappa R) \cosh(\kappa R) - \kappa R \} / 2\kappa^3 R^3$. Using $\sinh(\kappa R) \approx \cosh(\kappa R) = \frac{1}{2} e^{\kappa R} \gg \kappa R$ for $\kappa R \gg 1$, the ratio of central density ρ_C and surface density ρ_s to mean density $\bar{\rho}$ is approximately:

$$\frac{\rho_C}{\bar{\rho}} \approx \frac{8}{3} \kappa^3 R^3 e^{-2\kappa R}; \quad \frac{\rho_s}{\bar{\rho}} \approx \frac{2}{3} \kappa R \quad (4)$$

which hold to better than 1% for $\kappa R \geq 5$. Density profiles relative to mean density are sketched in Fig. 1 for $\kappa R = 5, 10$ and 20 . At the centre $r = 0$, all profiles are horizontal, as required for a physically acceptable solution. By assumption, the whole interior region $r < R$ is isobaric and therefore force-free. Binding forces from negative potential energy only appear as stresses on the surface $r = R$, where the density abruptly returns to atmospheric conditions over a small distance Δr . In the limit $\Delta r \rightarrow 0$, this discontinuity in ρ results in surface stresses tending to infinity as $1/(\Delta r)^3$, by equation (2). A pressure discontinuity maintains hydrostatic equilibrium at $r = R$. Inasmuch surface temperature as well as surface density are above atmospheric

conditions, interior pressures must be above 1 atmosphere. Low- κ solutions with little excess pressure should represent a low-energy ball lightning with restricted radiative output, as seen by Jennison. Other ball lightning reports specify internal energies in the range 10^6 – 10^8 (ref. 8), and a scorching radiative output of 4.5×10^5 W (ref. 14). This type should correspond to the high- κ limit of equation (4), where severe density stratification and high surface temperatures imply huge internal pressures. Already for moderate values of κ , the central density and temperature calculated from equation (4) fall in the region of thermonuclear interest. Table 1 gives a possible range of density, temperature and pressure for the case $\kappa R = 9$. Mean density $\bar{\rho} = 1.3 \text{ kg m}^{-3}$ and surface temperature $T_s = 4,000$ K served as reference for the calculation. The surface layer was treated as an ideal gas with atomic weight 14.2, and the central plasma was taken as fully ionised air.

Table 1 Range of parameters derived from hydrostatic equilibrium in an isobaric ball lightning model with $\kappa R = 9$

Parameter	Centre	Surface
Density	$\rho_C = 3.8 \times 10^{-5} \text{ kg m}^{-3}$	$\rho_s = 7.8 \text{ kg m}^{-3}$
Temperature	$T_C = 1.0 \times 10^8 \text{ K}$	$T_s = 4.0 \times 10^3 \text{ K}$
Pressure	$p = 181 \text{ atm}$	$p = 181 \text{ atm}$

The hydrostatic temperature profile of Table 1 is bound to be smoothed by energy transfer from the hot central region to the cooler surface layers. The mode of charge transfer and its effect on the equilibrium conditions in the ball lightning model are charged vortices in a turbulent plasma with: (1) electrical properties of a superconductor; (2) binding energy from density stratification; (3) energy storage in the pressure field.

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Electron-optical observations of ordered FeNi in the Estherville meteorite

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Scott and Clarke¹, using optical techniques, have recently reported the presence of ordered FeNi (taenite) with the tetragonal L1₂ structure (AuCu) in over 40 meteorites. The ordered phase occurs in 10–60 μm diameter regions and in 1–5 μm wide rims of clear taenite containing 48–57% Ni (refs 1, 2). In contrast with disordered γ Fe-Ni, the ordered taenite is optically anisotropic. The X-ray diffraction pattern and Mössbauer spectrum of ordered FeNi^{3–5} are also similar to that of tetragonal FeNi synthesised using high flux neutron irradiation at 300 °C of a disordered FeNi alloy in the presence of a magnetic field.

Although Mössbauer and X-ray diffraction studies^{6,7} have been used to detect superstructure in bulk samples, these techniques cannot spatially locate the ordered phase within the microstructure. The present study uses various electron-optical techniques, including transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM) and electron probe microanalysis (EPMA) to characterise and to resolve the ordered FeNi phase spatially. In addition, the first TEM evidence (both electron diffraction patterns and centred dark field (CDF) images) for superstructure in clear taenite, is presented.

A thin section of the Estherville meteorite containing a large area of continuous anisotropic taenite ($240 \times 500 \mu\text{m}$) was obtained from the Museum of Natural History, Smithsonian Institution, Washington, DC. The thin section also contains other areas that show optically anisotropic taenite in the form of $5 \mu\text{m}$ wide borders or as small patches that are often associated with schreibersite. Under crossed polars the large taenite field (Fig. 1) shows an intricate network of sub-domains and an irregular granular structure which is characteristic of anisotropic clear taenite^{1,2}. The EPMA trace (Fig. 1 inset) shows an approximately uniform composition across the taenite field. The average Ni content of the taenite is $50 \pm 2\%$ and this value is within the range 48–57% obtained for clear taenite^{1,2}.

Subsequent to the optical and EPMA studies the ($240 \times 500 \mu\text{m}$) clear taenite was extracted from the thin section for TEM and STEM investigations. Adopting a procedure outlined by Nord and James⁸, the clear taenite area was thinned to electron transparency by argon ion bombardment. The electron transparent thin foil was examined in a Philips EM300 electron microscope operating at 100 kV. Using a STEM attachment and X-ray energy dispersive spectrometry (EDS) localised chemical compositions were measured with an X-ray spatial resolution $\leq 50 \text{ nm}$.

TEM examination of the thin foil reveals areas that show characteristically different ion etching behaviour. Several 1–3 μm diameter preferentially thinned regions are observed in a

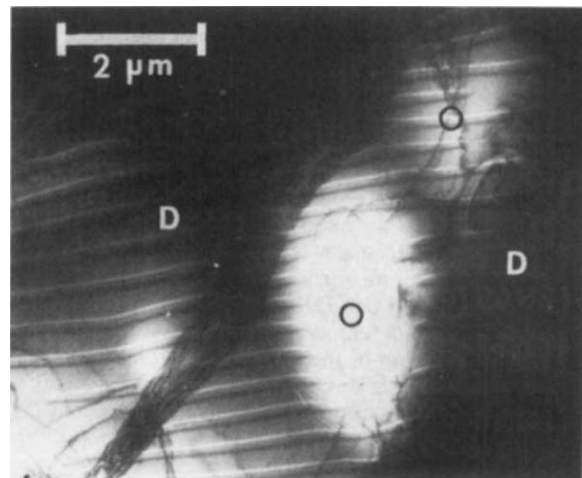


Fig. 2 Low magnification transmission electron micrograph of the ion thinned foil of clear taenite shown in Fig. 1. Notice preferentially thinned ordered regions (O) surrounded by disordered, or different ordered variant matrix (D). Lamellar features crossing both regions are magnetic domain walls imaged in slightly defocused conditions.

relatively thick matrix (Fig. 2). As the clear taenite is chemically homogeneous we suggest that the preferential ion etching of certain areas within clear taenite is structural rather than chemical in origin. The parallel black and white lines in Fig. 2 are characteristic of magnetic domain walls imaged in the TEM, and indicate the ferromagnetic nature of both the preferentially thinned and thicker areas.

Electron diffraction from several $20 \mu\text{m}$ selected areas indicates that the thin foil is a single crystal of taenite. In bright field images various structural defects are observed. These defects have the characteristics of twin boundaries, Neumann bands and a dense dislocation substructure. They are concentrated in the matrix regions and probably formed as a result of shock.

Electron diffraction patterns from all preferentially thinned regions show superlattice reflections in the fundamental FeNi

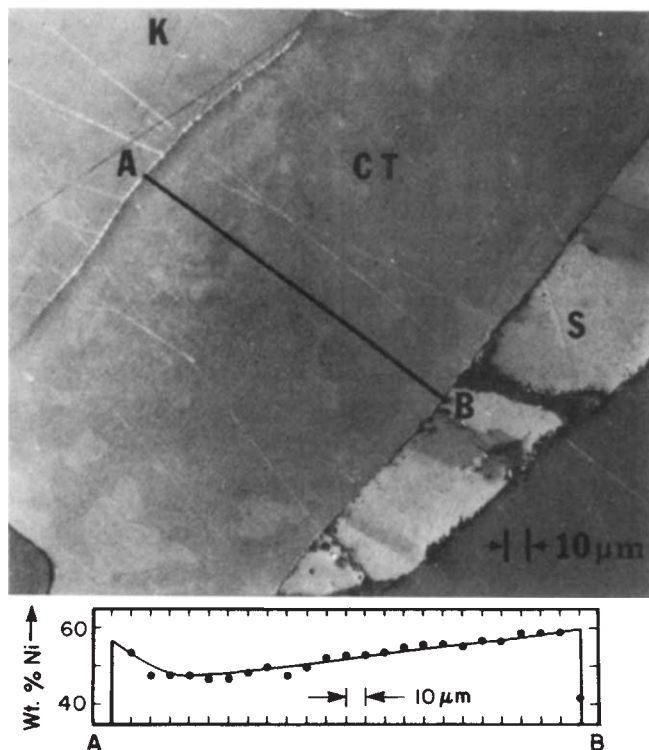


Fig. 1 Crossed polar reflected light photomicrograph of clear taenite (CT) surrounded by sulphide (S) and kamacite (K) in the Estherville meteorite. Notice the optical anisotropy (indicated by regions of contrast) in clear taenite. Electron probe Ni concentration profile taken across clear taenite is shown in the inset.

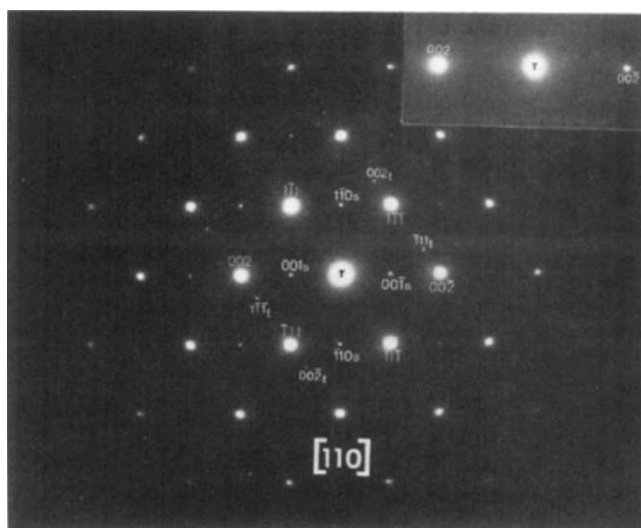


Fig. 3 Electron diffraction pattern taken from a preferentially thinned region in clear taenite (Fig. 2). The beam direction is $[110]$. The spot marked T is the transmitted spot, those marked S are the superlattice spots. The pattern also shows twin spots marked t which identify the twinning in the matrix as the conventional f.c.c. variety with the twin plane $K_1 = (1\bar{1}1)$. Note the absence of superlattice spots in the inset pattern taken from the adjacent matrix.

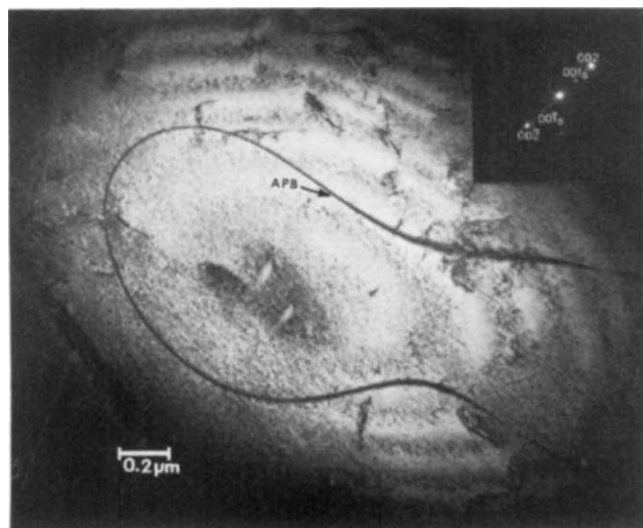


Fig. 4 Antiphase domain boundary (APB) in the ordered region of clear taenite shown in Fig. 2. Centred dark field image with (001) superlattice reflection. The accelerating voltage is 100 kV. The corresponding electron diffraction pattern is shown in the inset.

(f.c.c.) reciprocal lattice. The superlattice reflections arise from long-range ordering in these regions and indices can be assigned to these reflections on the basis of the LL_0 tetragonal structure. Figure 3 is a symmetric (110) zone axis pattern from an ordered region clearly showing (001) and (110) superlattice reflections. Absence of ordering in the adjacent matrix regions is indicated by disappearance of the superlattice spots in the diffraction pattern (Fig. 3, inset). (The electron diffraction pattern also shows faint twin spots which obey the conventional f.c.c. twinning law with (111) as the twinning plane. Twins were present across both ordered and disordered regions.) As a further confirmation of ordering, CDF images using superlattice spots were used to show the presence of antiphase domain boundaries (APB) within the ordered regions. Figure 4 shows a CDF image obtained with the (001) superlattice reflection. An APB shows up as a dark fringe running across the foil surface in the form of an open loop⁹. We propose, therefore, that clear taenite in the Estherville meteorite contains regions of ordered FeNi phase in a disordered γ matrix. The adjacent areas showing no superlattice spots are either disordered or alternatively (E. R. D. Scott, personal communication) are ordered LL_0 domains in which the {001} ordered planes are in another of the three possible, mutually perpendicular, orientations that could exist and hence are not correctly oriented for diffraction. It was not possible to distinguish between these two explanations since the specimen would have to be tilted to either a (100) or another (011) orientation to observe a different set of superlattice spots. In the natural orientation of this specimen this was not possible. STEM X-ray microanalysis from the ordered and adjacent regions indicates that both have the composition 52+2% Ni.

Evidence for the presence of coexisting ordered and disordered taenites in other meteorites has also been provided by Mössbauer and X-ray diffraction studies of Albertsen *et al.*⁴. However, their study shows 28% Ni content for the two taenites in which the disordered taenite is paramagnetic. This composition is clearly below that range obtained for clear taenite^{1,2}. More recently, electron microscopy of cloudy taenite in Santa Catharina meteorite by Jago¹¹ revealed two taenites associated with kamacite in a structure which apparently is a precursor to cloudy taenite. Jago¹¹ further reports that one of the taenites became ordered on heating to 400 °C.

We have presented direct TEM evidence for ordering of FeNi in clear taenite. No evidence was obtained for any crystallographic discontinuity between ordered regions and their surrounds. Therefore description of the optical anisotropy in terms of 'ordered grains' (refs 1, 2) is considered inaccurate. There is

also a clear distinction between TEM and Mössbauer results, since in the latter there is no evidence for the presence of both ferromagnetic taenite and ordered FeNi in the same taenite field⁴. This inconsistency must be explored further. The ordered phase may have formed when the parent meteorite body cooled below the ordering temperature of the superstructure ($T_c = 320$ °C) as suggested by Danon *et al.*¹⁰.

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Determination of crystal structure of metastable anthracene by a novel method

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A combination of transmission electron microscopy (TEM) and 'atom-atom' computational procedures^{1,2} developed for molecular crystals has led to the discovery and the determination of the crystal structure and lattice dynamics of a metastable, triclinic phase (hereafter designated, II) of anthracene. The new form, which is only 2 kJ mol⁻¹ less stable than the monoclinic, thermodynamically stable parent phase (I), may be generated by the application of compressive force or shear stress approximately perpendicular to the basal plane of I. In essence, the size and shape of the unit cell into which molecules of anthracene are packed is determined from the electron diffraction patterns of the new phase. From the symmetry and space group, determined by TEM, assuming that the individual molecules are as undistorted in II as in I, a 'trial structure' of the 'new' phase may be computed by minimising the total energy as a function of the molecular packing characteristics, knowing the empirically determined³ atom-atom potentials between all pairs of non-bonded atoms. The structure is refined by varying the molecular coordinates, as described below, so as to arrive at a set of lattice vibrations^{4,5} all of which are real in the entire Brillouin zone. The enhanced photo reactivity of phase II compared with I is explicable in terms of the resulting crystal structure.

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We recently reported⁶ the facile production of a metastable phase of anthracene which was discovered from analysis of the electron diffraction patterns of anthracene (I) that had suffered stress at room temperature. Phase II is produced topotactically⁷ inside I such that $(001)_{\text{II}} \parallel (001)_{\text{I}}$, $(100)_{\text{II}} \parallel (\bar{1}01)_{\text{I}}$ and $(010)_{\text{II}} \parallel (011)_{\text{I}}$. Moreover, the conversion of I leads to a coexistent bi-phasic matrix of I and II; irradiation of this matrix with UV light rapidly results in the solid state formation of dipara-anthracene (by formation of $C_9-C'_9$ and $C_{10}-C'_{10}$ single bonds at the 'meso' positions of neighbouring molecules).

From the topotactic relationships quoted above, a set of unit-cell dimensions for II may be derived: $a = 8.56$, $b = 6.04$ and $c = 11.22$ Å, $\alpha = 122.6$, $\beta = 101.2$ and $\gamma = 90.0^\circ$ (in terms of the reported crystal structure⁸ of I). But when the standard energy minimisation procedure using appropriate³ 'non-bonded' potentials of the form: $U_{ij} = A/r_{ij}^6 + B \exp(-Cr_{ij})$, was used the resulting structure, although it had a lattice energy only marginally less than that of I, possessed imaginary frequencies for a few lattice vibrations, thereby indicating that this trial structure was untenable. We therefore explored the energetics and dynamics of a range of structures based on slight distortions of this trial structure. We set an upper-limit of $\pm 5\%$ for the degree of mismatch between the cell dimensions of I and II—a figure which is reasonable in view of the absence of observed electron-microscope strain contrast at interfaces of II and I. From the many sets of unit-cell dimensions for II that may be generated on this basis there emerged, after packing analysis (energy minimisation) and lattice dynamical calculations, either: (1) dynamically unstable structure(s); or (2) the stable monoclinic $P2_1/a$ phase (I) or (3) a triclinic structure (space group $P\bar{1}$, $Z = 2$ and cell constants $a = 8.342$, $b = 5.892$, $c = 11.282$ Å, $\alpha = 123.34$, $\beta = 96.70$ and $\gamma = 85.91^\circ$) with no imaginary lattice frequencies (the minimum energy structure quoted in ref. 6 has subsequently been found to be dynamically unstable). Full details of the structure are given elsewhere⁹, but the salient difference is the intermolecular disposition of the two molecules situated at $(1, 0, 0)$ and $(\frac{1}{2}, \frac{1}{2}, 0)$ as shown in Fig. 1. This new phase II, which does not seem to exist in the bulk phase, readily reverts to phase I in normal conditions of temperature and pressure: at low temperatures its lifetime may be prolonged. As the temporary stabilisation of II could arise from the presence of the topotactic interfaces with I, we have also looked at their energetics and structure using the non-bonded potentials and the energy minimisation procedure.

Using a computational approach in which layers of I and II on either side of an imaginary interfacial plane are in coherent contact (zero mismatch), with lateral mismatches progressively increasing with distance away from the interface, it is possible to estimate the 'excess energy' at the three principal boundary planes that give rise to the topotaxy. Again a pairwise evaluation of non-bonded interactions is undertaken. The 'excess energies' at all three topotactic interfaces are small (roughly a tenth of the surface energy of the respective plane), unlike the typical excess energies at high- and low-angle boundaries in metals (which are, respectively, about a third and a sixth of the corresponding surface energies). Besides the favourable energetics at the interface, we also find that the intermolecular contacts and molecular conformation across the interfaces are analogous to those found within the bulk structures of I or II. These results are in line with our observations in the electron microscope where we have found no direct evidence for mismatch dislocations, strains or clear boundaries between the two phases.

Careful control over the structural and chemical purity of the anthracene crystals and subsequent photo-reaction studies (in the TEM), reveal unmistakably the positive influence of the new phase on dimer formation. Enhanced photoreactivity is exhibited at the compressed regions of the crystal.

Significantly, compared with the situation that prevails for phase I, some of the intermolecular dispositions are different in phase II and at interfaces of I and II. Of particular relevance is the shortening of both the $C_9-C'_9$ and $C_{10}-C'_{10}$ distances between the adjacent molecules $(1, 0, 0)$ and $(\frac{1}{2}, \frac{1}{2}, 0)$ on proceeding from I

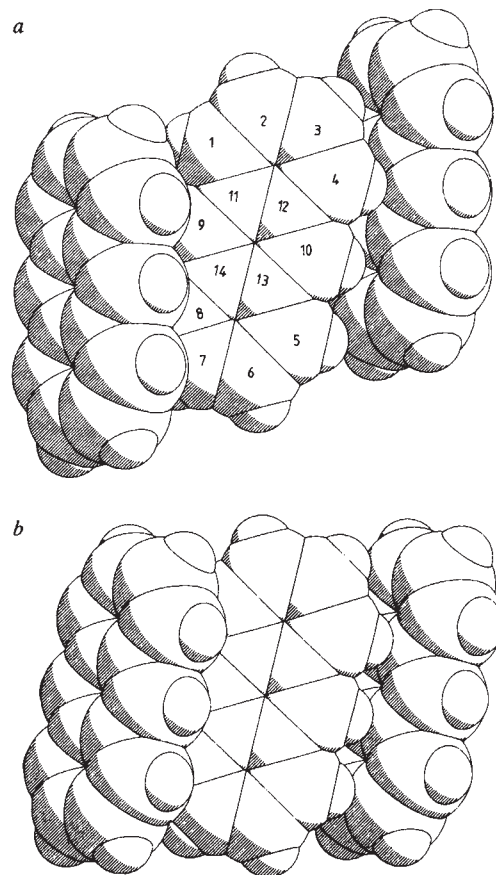


Fig. 1 Illustration of mutual orientation of nearest molecules in phases I (a) and II (b) or crystalline anthracene. Only three molecules in the (110) plane are shown.

to II. The green fluorescence observed in the compressed specimens may also be attributed to the slightly increased overlap between these molecules in II. Also of relevance is the relative ease with which a molecule of anthracene at $(\frac{1}{2}, \frac{1}{2}, 0)$ in phase II may be rotated (with limited hindrance from surrounding molecules) about its long axis, thereby bringing it face-to-face and overlapped with its adjacent molecule at $(1, 0, 0)$. Both these factors will render II more susceptible than I to photodimerisation, in line with observation.

Thus, the present method can be used to elucidate structural information from basic TEM data, starting with a static calculation procedure (lattice-energy minimization) followed by a further refinement using a dynamic treatment (phonon dispersion). Notwithstanding the limits imposed by the approximations inherent in the calculation methods used, it is anticipated that this approach to crystal structure determination should be widely applicable to other organic crystals, especially where problems of small crystal size and the occurrence of polymorphic intergrowths are encountered.

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Polar ice evidence that atmospheric CO₂ 20,000yr BP was 50% of present

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Analysis of the air enclosed in polar ice (~0.1 ml per g of ice¹) provides one of the most promising ways of discovering atmospheric composition over the past 100,000 yr. Until now, because of an apparent but not well understood enrichment of CO₂ in the trapped air, all attempts to reconstitute the ancient atmospheric CO₂ content from polar ice have failed²⁻⁶. We have obtained CO₂ contents that can reasonably be considered representative of present or past atmospheric contents using a new method of air extraction. The results reported here, based on the CO₂ analysis of two deep Antarctic cores including the last climatic interchange, strongly suggest that during the coldest part of the last Ice Age (20,000–15,000 yr ago) the atmospheric CO₂ content was half (0.016%) that of today's level (0.033%).

Carbon dioxide extracted in a vacuum from melted ice samples can originate from: (1) air contained in bubbles within the ice; (2) gas which may be trapped or adsorbed by the ice lattice; or (3) the decomposition of dissolved carbonates or carbonate particles which are present naturally or artificially (through contamination) in the solid.

Raynaud and Delmas have previously determined the CO₂ content of ice cores from several polar sites (Greenland and Antarctica) and of various ages (Holocene and Wisconsin periods)⁵. All these determinations showed a large CO₂ enrichment with respect to the present-day composition. The general mean was 0.35% at Camp Century (Greenland) and 0.13% at Byrd Station (Antarctica). We concluded that the gas-solid interaction in the firn is probably responsible for this phenomenon. However, we pointed out that a modification of the initial CO₂ content during storage of the ice core could not be excluded. More recent results⁷ concerning the detailed analysis of an ice core representing ~2 yr precipitation at Camp Century also show a high enrichment of CO₂ (content, 0.1%) in the extracted air.

Table 1 CO₂ in Antarctic ice (% in the total gas extracted)

Site	Depth (m)	No decontamination	% of CO ₂ Decontaminated	Decontamination method
D10	113	0.61 (1)	0.034 (6)	b
66°40' S, 140°01' E	148		0.036 (4)	b
270 m, -19 °C, 0.123	226		0.032 (5)	b+d
	226		0.021 (2)	b
	248		0.038 (1)	b
Dome Summit	100	0.13 (32)	0.037 (1)	b
66°17' S, 110°32' E				
1,390 m, -22 °C, 0.115				
Dome C (DC)	114		0.042 (2)	a
74°39' S, 124°10' E	137		0.037 (3)	b+c+e
3,240 m, -53 °C, 0.0845	249	0.32 (1)		
	300	0.28 (1)		
	330		0.054 (1)	a
	473	0.22 (1)	0.074 (1)	b
	473		0.050 (2)	a
	589	0.105 (1)	0.051 (1)	a
	589		0.048 (1)	b
	670		0.067 (1)	a

Results without, and after decontamination of the ice (see text). For each site elevation (m), mean annual temperature (°C) and total gas content in the depth range 100–150 m are given (ml per g of ice) (from ref. 8). Number of determinations are given in parentheses. Decontamination methods: a, water, 20 °C; b, ethanol, -15 °C; c, acid bath, 20 °C; d, ultrasonic acid bath, 20 °C; e, acid bath -12 °C.

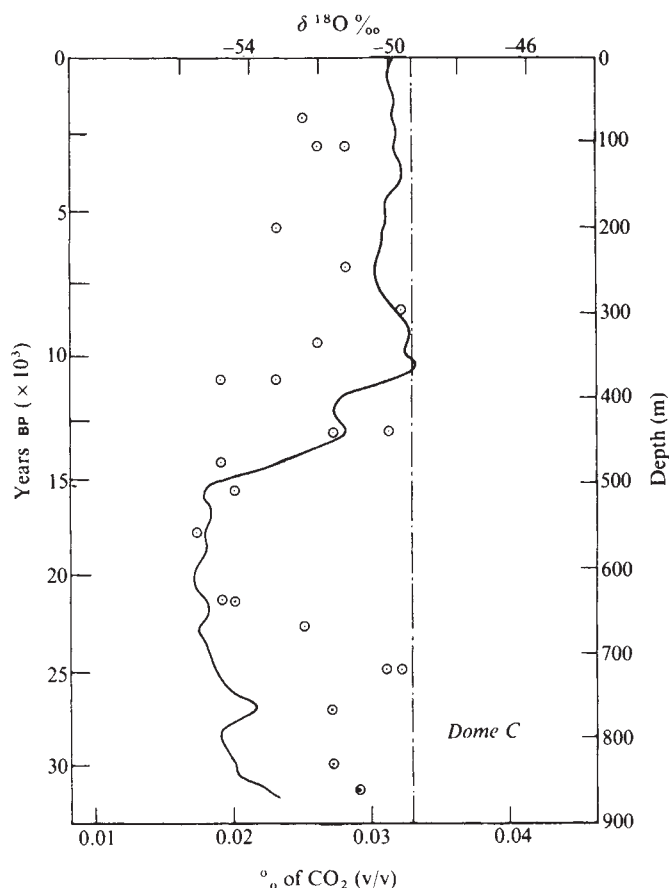


Fig. 1 % of CO₂ (v/v) in the air of the bubbles from the Dome C ice core against depth (in metres of ice equivalent). Isotopic values ($\delta^{18}\text{O}$ ‰ continuous line) and dating are as published previously¹⁰. Note the low CO₂ contents between 22,000 and 15,000 yr BP. The present day atmospheric CO₂ content (0.033%) is indicated by a dashed line.

Classical gas extraction procedures and CO₂ content determination have been described previously^{5,8}. The ice core is cut in a cold room with an electrical band saw. All faces of the sample (a cube of 30–50 g) are 'new' to minimise contamination. The sample is placed in a glass vessel which is then mounted in a vacuum line. After melting of ice, CO₂ content is determined in the extracted gas chromatographically. We have investigated first the contribution of carbonates (actually bicarbonates HCO₃⁻) which could be responsible for the CO₂ enrichment. On the basis of a 0.03% CO₂ content in the gas extracted, 1,000 g of ice contains ~30 µl of CO₂ whereas 1 µEquiv. of HCO₃⁻ releases through decomposition 22.4 µl of CO₂. This comparison shows that the total CO₂ measurements are very sensitive to the possible presence of small amounts of carbonates in the lattice. For 32 samples of Dome Summit (66°17'S, 110°32'E) for which the 'classical' gas extraction method gave a mean value of 0.13% of CO₂ ($s=0.07$), we determined, by a sensitive (± 0.2 µEquiv. 1⁻¹) titrimetric method, a surprisingly high mean bicarbonate content of 7 µEquiv. 1⁻¹. As estimation of the natural carbonate content leads to much smaller values, contamination of the samples could be responsible for this high carbonation. (The molar ratio HCO₃⁻/Na is 0.005 in sea salt, assumed to be the major impurity (some µEquiv. 1⁻¹) in snow at this coastal site.) By rinsing copiously the surface of the solid sample with double permutated water or ethylalcohol we considerably reduced the carbonation.

New determinations of CO₂ in decontaminated polar ice were made as for the carbonate measurements. The results (Table 1) show clearly that the gas extracted from the ice has a CO₂ content which is not significantly different from the present-day air composition. These results contrast strongly with the former reported values. Our experiments explain *a posteriori* the poor reproducibility of the previous CO₂ measurements, the

difficulty of extracting the total CO₂ from meltwater and the high pH values of the meltwater sometimes observed⁷.

To eliminate more completely the contamination problems linked with the ice lattice, we developed a dry extraction method. The solid ice sample (~40 g) was pulverised under vacuum at -40 °C in a closed stainless steel container with the aid of two metallic balls for 30 s. About 75% of the trapped gases are released. They were then transferred to the gas chromatograph, as for a wet extraction. We checked that our CO₂ concentration measurements were independent of grinding time, of gas extraction rate, as well as of the state of the sample (contaminated or fractured).

Two deep Antarctic ice cores have been analysed by this method (Dome C and D10). The results (Table 2) are plotted against depth and age in Figs 1 (Dome C) and 2 (D10). The average CO₂ concentration was 0.025% at both locations, that is, much less than published to date and still slightly lower than values obtained using the classical extraction method after decontamination (Table 1) particularly for the deepest fractured

Table 2 CO₂ content of ice air bubbles at DC and D10 (Antarctica)

DC			D10		
Depth (m)	% CO ₂	±σ (×10 ³)	Depth (m)	% CO ₂	±σ (×10 ³)
103	0.025	2.0	37	0.026	2.0
137	0.028	3.4	61	0.027	2.3
	0.026	3.2	113	0.033	2.7
232	0.023	2.0	148	0.034	2.7
277	0.028	2.8	154	0.032	2.5
330	0.032	2.7	191	0.035	2.8
366	0.026	3.0	208	0.032	3.3
410	0.023	2.6	218	0.017	1.6
	0.019	2.6	227	0.016	2.3
472	0.031	2.7	237	0.018	2.1
	0.027	2.8	247	0.021	2.5
507	0.019	2.6	285	0.020	2.3
541	0.020	2.8	301	0.019	2.3
589	0.017	2.6			
670	0.020	2.8	Overall mean value	0.025	0.72
	0.019	2.8			
701	0.025	3.6			
752	0.031	2.3			
	0.032	3.9			
800	0.027	4.0			
864	0.027	3.9			
893	0.029	4.1			
Overall mean value	0.025	0.46			

The standard deviation (s.d.) is given for 95 % confidence limits.

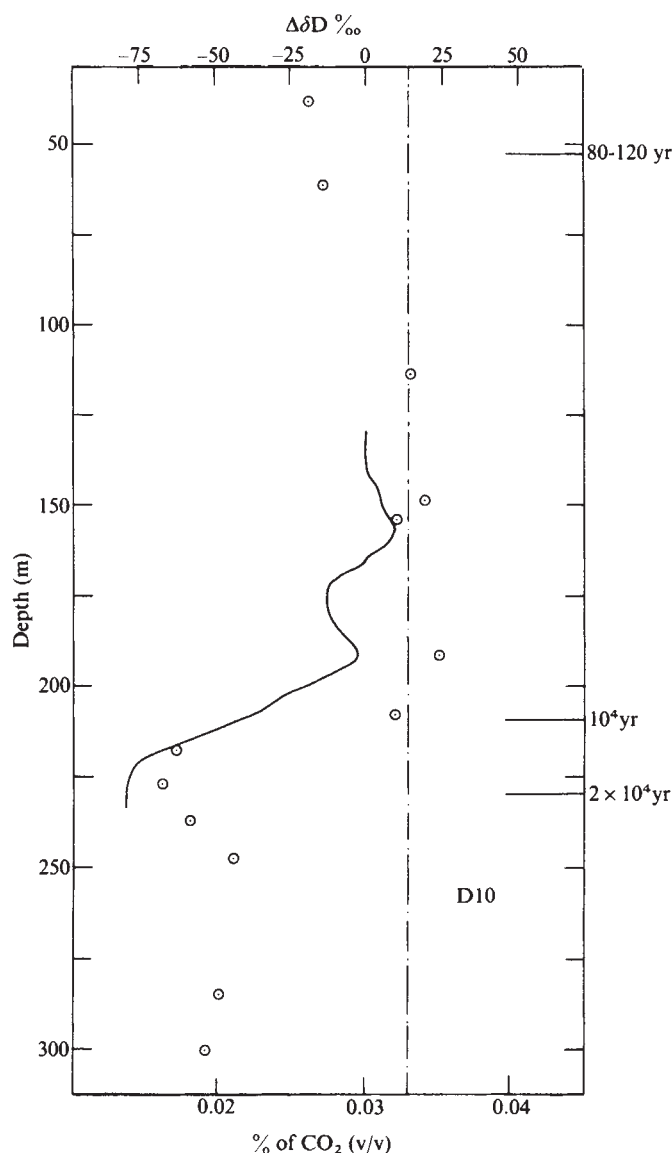


Fig. 2 % of CO₂ (v/v) in the air of the bubbles from the D10 ice core against real depth (in metres). The interpretation of the isotopic profile ($\delta D\%$, continuous line) is complicated in this coastal site by glacier flow problems. Climatic fluctuations are given here in terms of variations of $\delta D\%$ ($\delta D\%$). They have been reconstituted by Raynaud *et al.*⁹ and apparently include the last climatic interchange. Low CO₂ contents are observed during the coldest period (215–235 m). The present-day atmospheric CO₂ content (0.033 %) is indicated by a dashed line.

(and hence possibly contaminated) ice samples. In some cases (113 and 148 m, D10), nearly identical results were obtained (0.033–0.036%) by using either analytical method—a good indication that CO₂ is located essentially in the bubbles. On the other hand, the 'dry' extraction method seems to be the only one convenient for CO₂ analysis of fractured ice cores (even if they have lost important volumes of gases—as is the case for the two cores we studied from a depth below 200 m).

The most interesting feature in Fig. 1 is the marked CO₂ depletion (0.016–0.020%) during the coldest part of the glacial age (15,000–20,000 yr BP). The same is observed at D10, although the dates of the ice layers are still being questioned at this location. The temperature increase (estimated at DC to be ~7 °C (ref. 10)) which followed the end of this period is accompanied by a similar trend for CO₂. However, our results are not accurate enough to conclude whether the increase in atmospheric CO₂ content preceded or proceeded the climatic change. A time lag could exist between the age of the ice and the age of the entrapped gases because the interstitial air of the firm is probably in contact with the free atmosphere for a long time (up to 2,000 yr at Dome C). On the other hand the interstitial air could be also contaminated by older air expelled from deeper firm layers by the slow compaction process. The net result of such effects could be a smoothing of the variations of the initial atmospheric CO₂ content. During the Holocene, the mean values (0.031% at D10, 0.027% at DC) are relatively consistent and essentially in agreement with the estimated pre-industrial values (0.027–0.029%)^{11,12}. The variations of the CO₂ content during this period, particularly at Dome C (six depth levels studies), can be explained by the reported analytical uncertainties (Table 1). Nevertheless, at both locations, the highest CO₂ contents of the Holocene period are obtained around the climatic optimum which immediately follows the climatic interchange¹⁰. The results concerning the oldest samples (ages > 20,000 yr) are difficult to interpret due to dating uncertainties, especially at D10.

Finally although physicochemical processes within the firm (such as adsorption) could also affect the composition of the air trapped in the polar ice^{5,13}, these new results show that the importance of the CO₂ enrichment found in the polar ice gases has been largely overestimated and that the ancient variations of the atmospheric CO₂ content can most probably be reconstructed from deep ice cores.

Berner *et al.*¹⁴ have recently analysed two deep ice cores (Camp Century, Greenland and Byrd, Antarctica) for CO₂ content. Their experimental procedure for extracting CO₂ from the ice was similar to our 'classical gas extraction method'. They consider two different CO₂ fractions (the gaseous CO₂ and the CO₂ in the ice lattice), and their results strongly suggest that the atmospheric CO₂ concentration during the last glaciation could have been a factor of 1.5 lower than today, which is in good agreement with our own results.

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Recent climatic trends and local glacier margin fluctuations in West Greenland

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Recent climatic trends in the Arctic have been characterised by a general cooling between the mid-1950s and the late-1960s, followed by a return to warmer conditions in the early 1970s (refs 1, 2). Throughout the Canadian Arctic Archipelago and at Thule in north-west Greenland a marked decrease in summer temperature occurred after 1963, and winter precipitation increased^{3,4}. These changes were accompanied by a lowering of the average July freezing level height by as much as 500 m (ref. 5), decreased glacier mass loss⁴ and increased glaciation³. Here I report similar climatic trends in West Greenland and demonstrate different glacier responses, in particular an advance of cirque and small valley glaciers since about 1968, contrasting with a simultaneous retreat of larger valley and icefield outlet glaciers.

Mean annual and seasonal temperature and precipitation trends for Godthåb (64°10'N, 51°33'W) since 1920 are presented in Figs 1 and 2, based on data provided by the Meteorologisk Institut in Copenhagen. Summer is defined as the months May to September; winter as the months October to April. These groupings approximately encompass the glacier ablation and accumulation seasons, respectively. Spring and autumn are broadly equated with the months May and September, respectively. A major feature of the temperature graphs is the marked cooling in all seasons from about 1966 to 1971 followed by a trend towards warmer conditions after 1971, except in winter. During 1971, 5-yr mean summer temperatures reached their lowest level since the start of continuous records in 1875. Winter and summer precipitation increased sharply in the 1950s and

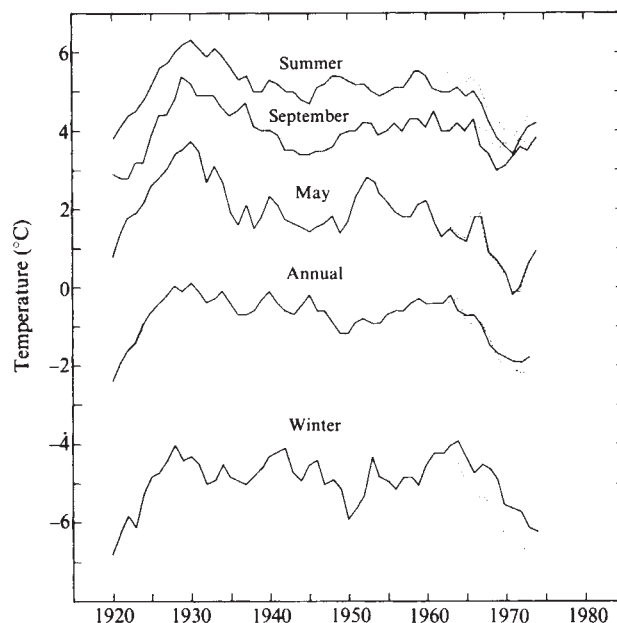


Fig. 1 Five-year running means of temperature at Godthåb (solid lines) and Sukkertoppen (dotted lines). Data are plotted for the middle year of each five year period. Winter data are plotted for the second year of each winter.

1960s, and the highest 5-yr means since the start of continuous records in 1920 were in 1966 and 1963, respectively. Thereafter, precipitation totals fell, but remained above those for the years 1920 to 1960. Climatic data for Sukkertoppen, 145 km north of Godthåb and the nearest town to the glaciers investigated, are only available from 1960 onwards, but the trends from this date are similar to those for Godthåb (Figs 1 and 2). The origin of the above trends has been related to the development and persistence of a ridge of pressure anomaly over Greenland in the 1950s and 1960s and its sudden collapse after the winter of 1970–71². Manifestations of the climatic deterioration in the form of increased ice-cover of the sea and biological changes have already been reported^{1,2}. I now describe some glaciological effects.

The area at the head of Íkamut kangerdluarssuat (Fig. 3), 45 km north of Sukkertoppen, is one of spectacular alpine-type glaciation: many cirque and small valley glaciers up to 6 km long

Table 1 Glacier locations, sizes and icefront changes

Glacier	Location		Length (km)	Overall change in glacier front position between 19th century ice maximum extent and 1968–69 (m)	Overall change in glacier front position between 1968–69 and 1978 (m)
	lat	long			
1	65°49'36" N	52°39'47" W	3.5	–1,260	+82
2	65°45'20" N	52°40' 7" W	2.3	–480	+50
3	65°46'41" N	52°46'57" W	1.5	–540	+46
4	65°47' 0" N	52°50'52" W	2.2	–730	+20
5	65°46'21" N	52°34' 5" W	2.5	–690	+117
6	65°48' 6" N	52°34'44" W	6.0	–1,200	–34
7	65°41'42" N	52°37'24" W	2.0	–800	+158
8	65°42'26" N	52°36' 6" W	10.0	–1,500	–190
9	65°44' 8" N	52°35'56" W	7.5	–1,180	–55

Glaciers 1 to 7 are cirque or valley glaciers; glaciers 8 and 9 icefield outlet glaciers.

are set among mountains rising steeply from sea level to a maximum altitude of 1,755 m, and several outlet glaciers drain an icefield approximately 40 km by 15 km in size at an altitude of about 1,200 m above sea level and existing independently of the Inland Ice. Before about 1968 the glaciers of the Sukkertoppen area had been progressively retreating, although interrupted by several short halts, from the advanced positions of their historical maximum extent in the mid to late nineteenth century⁶. This pattern has been confirmed for seven cirque and valley glaciers and two outlet glaciers investigated during the summer of 1978⁷. The positions of these glaciers in relation to prominent points in their forelands were compared with those on vertical aerial photographs taken in 1968 in some cases and 1969 in others by the Geodætisk Institut, Copenhagen. The two outlet glaciers and the largest valley glacier have continued to recede overall between 1968–69 and 1978 by between 34 and 190 m (Table 1). However, in two of the forelands (6 and 9 in Table 1) the presence of end moraine ridges between the 1968–69 and 1978 ice margin positions indicates temporary reactivation at some stage during this period. In the case of glacier 9 the ridge is 3.5 m high, and the volume of debris clearly represents several years accumulation when compared with that seen melting out from the glacier in 1978. As two recessional ridges superimposed on its proximal slopes are probably push moraines formed during the winters of 1977–78 and 1976–77, I infer that the main ridge was formed between about 1971 and 1975. It is unlikely to have been formed much earlier as it lies inside the 1969 ice margin position. The glacier has since receded by up to 10 m from the main ridge crest. Neither plants nor lichen were observed in the immediate proglacial areas of the three receding glaciers.

In contrast, six of the cirque and valley glaciers have advanced overall from their 1968 or 1969 positions by distances of between 20 and 158 m (Table 1). When visited, they were generally in close contact with fresh moraine and boulder ridges at their margins, and the advance was probably still in progress. In two cases slight recession of 1 to 2 m from the ridges may

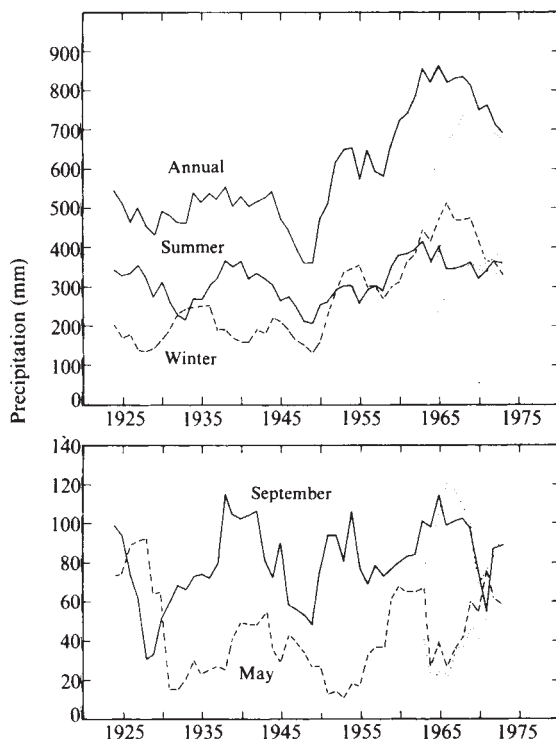


Fig. 2 Five-year running means of precipitation at Godthåb (annual, summer and September, solid lines; winter and May, dashed lines) and Sukkertoppen (dotted lines). Data are plotted for the middle year of each 5-yr period. Winter data are plotted for the second year of each winter.

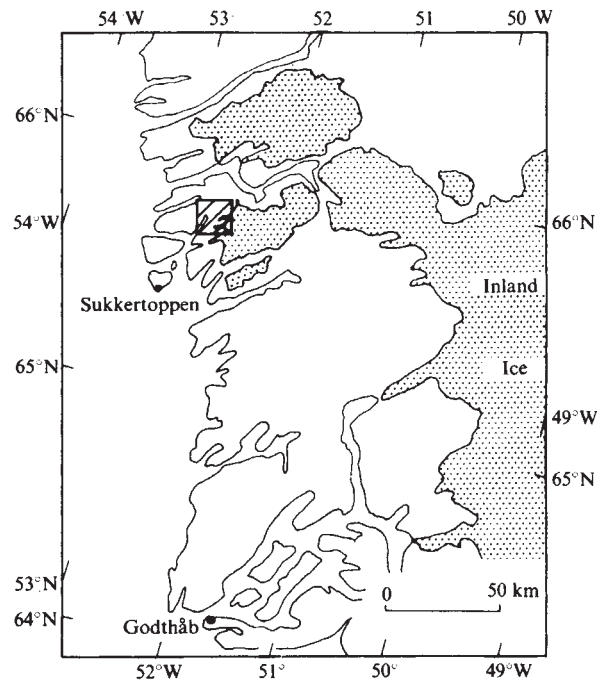


Fig. 3 Location map. The glaciers investigated occur within the shaded square north of Sukkertoppen. Major glaciers only are shown by stippling.

reflect negative summer balances as observations were not made at the start of the ablation season. Plants and patches of moss were often incorporated or disturbed at the distal edges of the marginal debris ridges. Lichen of *Rhizocarpon* species with thalli up to 9 mm in diameter (equivalent to an estimated age of 22 yr) occurred within 15 m of some of the glacier fronts.

The recent frontal changes of the cirque and small valley glaciers are sufficiently similar in direction and timing to argue that they relate to the changing climatic conditions rather than internal instabilities or surges. I therefore comment on possible relationships between the climatic trends and the general response of the glaciers. Theoretically the glaciers will respond relatively rapidly to changes in temperature or radiation conditions because of the immediate effects on ablation in the sensitive snout areas, but there will tend to be a lag in their response to changing precipitation conditions while the effects are propagated down-glacier from the accumulation areas.

The advance of the cirque and small valley glaciers since about 1968 coincides in its first part with a period of decreased summer temperatures and a trend towards colder, wetter springs and colder, drier autumns, and can be seen as a direct response to this climatic deterioration. However, despite a reversal in all these trends after 1971, the glaciers have continued to advance at least until 1978. Therefore, the observed pattern of glacier margin fluctuations matches only in part the recent temperature trends. The continued advance of the glaciers probably reflects a lag in their response to the increased precipitation during the late 1950s and 1960s. The magnitude of this lag is at least 9 yr judging from the fact that precipitation began to decrease sharply after 1969.

The temporary reactivation interrupting the continued recession of one of the outlet glaciers and the largest valley glacier can be explained as a direct response to the climatic cooling, particularly in summer seasons during the late 1960s and early 1970s. However, these glaciers do not seem to have responded significantly to the increased precipitation in the 1950s and 1960s. This could reflect either a lag or a damping of any effects due to the size of the glaciers. The former is more probable because of the magnitude and duration of the precipitation increase. The continued recession of the two outlet

and the largest valley glaciers at present can be explained partly as a direct response to the recent climatic warming and partly as a lag response to the relatively low precipitation during the 1940s and early 1950s. If this is the case, then a minimum lag of 20 to 30 yr in the response of these glaciers to precipitation changes is implied.

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Early Flandrian land and sea-level changes in Lower Strathearn

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The morphological and stratigraphic studies reported here relate to early Flandrian relative sea-level changes in the carselands, or Postglacial raised estuarine flats, of Lower Strathearn. These data coupled with the study and dating of associated environmental changes by pollen and diatom analysis and radiocarbon assay, have enabled graphs of relative sea-level changes and land uplift to be constructed.

The carselands are backed by the Main Postglacial raised shoreline, whose local altitude is 9.8–10.2 m OD (ref. 1), and whose ^{14}C age has been determined elsewhere in east-central Scotland to be in the range 5,900–7,200 yr BP (refs 2–4). The carse deposits, consisting of estuarine silt and clay, vary in thickness from a thin veneer to well over 10 m, and are extensively underlain by a bed of terrestrial peat, which in turn rests on sandy estuarine deposits. The surface of the latter is shown by more than 250 boreholes sunk by the authors to form a staircase of buried steps separated from each other by distinct bluffs, the foot of each bluff representing a buried raised shoreline.

These morphological and stratigraphic relationships, which are broadly similar to those established in the Forth carselands^{5,6}, suggest the following sequence. A Lateglacial and early Flandrian phase of generally falling relative sea level was punctuated by stillstands and/or transgressive episodes resulting in the formation of what are now buried raised estuarine flats in descending order of age and altitude, the abandonment of the flats being followed by the growth of vegetation, including peat. Later, relative sea level rose again, causing the progressive burial of the peat-covered flats, in ascending order, by the carse deposits, and culminating in the formation of the extensive carseland surface visible today.

The 15 ^{14}C dates from six sites used in constructing the relative sea-level and land uplift curves (Figs 2 and 3) are listed in Table 1, and the locations of the sites are shown in Fig. 1. The data include two ^{14}C dates relating to the culmination of the Main Postglacial transgression at Glencarse⁷ (site 6), in the Carse of Gowrie, as this site is close to, and lies on the same Main Postglacial Shoreline isobase as, the Carey–Cordon area¹.

At Carey (site 1, Fig. 1) a 59 cm-thick bed of highly-compressed peat rests on medium sand, and is overlain by >6 m of carse deposits. The pollen record established by analysis of samples at 1–2 cm intervals throughout the peat and the upper 7 cm and lower 6 cm of underlying and overlying estuarine sediments contains no suggestion of any gaps in the depositional record. Although few unambiguous indicators of a salt-marsh environment are present, a reed-swamp succession may be suggested, especially at the basal transition, by the high frequencies of Gramineae pollen (up to 70% of 500 total land pollen). These are interpreted as reflecting the presence of *Phragmites*, of which there is also macroscopic evidence. The diatom assemblage of the basal 12 cm of carse deposits is dominated by several species of *Fragilaria*, averaging >70% of the total count (1,000 valves). In common with sites 2–5, diatoms are abundant only within and above the peat-carse transition, and the buried estuarine deposits contain only small numbers, which include both marine and brackish-water taxa. The base of the peat (altitude 3.2 m OD) at two different places along the same exposure has been dated at $9,640 \pm 140$ yr BP (I-2796)^{1,9} and $9,524 \pm 67$ yr BP (SRR-72)⁸, giving the approximate date of initiation of peat growth following the withdrawal of estuarine conditions (point 1a, Fig. 2). Pollen evidence confirms this date, with *Betula*, *Juniperus* and *Filipendula* all present in the basal peat samples, as has been found at Main Buried Beach sites in the Forth valley^{10,11}. As the Carey exposure is located close to the buried shoreline (altitude 3.2 m OD) the ^{14}C dates must relate closely to the abandonment of the latter, especially the one derived from the basal 1 cm of peat (I-2796). The Carey buried shoreline is believed to correlate with the Main Buried Shoreline of the Forth valley^{5,6}, which has been similarly dated at about 9,600 yr BP. (J. B. Sissons, personal communication). The top of the peat at Carey (present altitude 3.8 m OD) gave radiocarbon ages of $7,605 \pm 180$ yr BP (NPL-127)^{1,9} and $7,778 \pm 55$ yr BP (SRR-71)⁸, dating the onset of peat burial beneath the carse deposits (1b, Fig. 2). (Note that in ref. 9, the I-2796 date was erroneously recorded in yr BC instead of yr BP.)

At Innernethy (site 2) an 80 cm-thick peat bed rests on the silty sand of an extensive buried estuarine flat. The latter is next below the Carey feature in the staircase of buried flats, and site 2 is located adjacent to the buried shoreline (altitude 2.8 m OD). A 1 cm-thick peat band that occurs within the buried estuarine deposits, 18 cm below the base of the main peat bed, demonstrates that the buried shoreline was formed at the culmination of a transgression. The ^{14}C ages of this thin peat band (2a, Fig. 2; altitude 2.7 m OD) and the base of the main peat bed (2b; altitude 2.8 m OD) date this culmination to between $8,555 \pm 60$

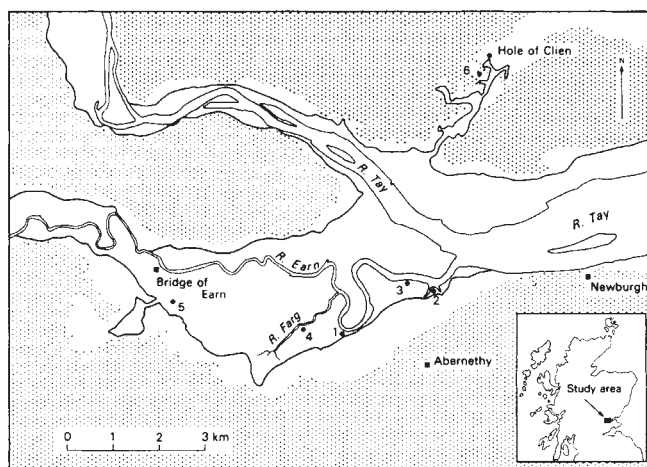


Fig. 1 Location of sites: 1, Carey; 2, Innernethy; 3, Cordon; 4, Culfargie; 5, Kintillo; 6, Glencarse. The heavy, continuous line represents the Main Postglacial Shoreline, marking the inner edge of the carselands. Land above 15 m OD is stippled.

(SRR-1399) and $8,505 \pm 50$ yr BP (SRR-1398). These dates are statistically indistinguishable at the 95% level from the only published date of $8,690 \pm 140$ yr BP (I-1839)^{4,6,10} relating to the Low Buried Beach in the Forth valley, with which the Innerneath buried flat should correlate by morphological analogy. This may imply that the Low Buried Shoreline was abandoned about 8,500 or 8,600 ^{14}C yr ago, rather than the 8,800 yr usually quoted^{6,13}, a possibility that is reinforced by the basal peat date at site 3. Apart from a brief appearance of *Chenopodiaceae* corresponding to the thin peat band within the buried estuarine deposits, the only apparent vegetational succession is at the peat-carse boundary, where a sequence of freshwater environments may be indicated before the onset of carse deposition (2c; present altitude 3.6 m OD) at about $7,530 \pm 50$ yr BP (SRR-1397). The diatom record in the carse deposits suggests marine and brackish water conditions, as indicated by *Paralia sulcata* (Ehrenberg) Cleve, *Rhaphoneis surirella* (Ehrenberg) Grunow ex Van Heurck and *R. amphiceros* (Ehrenberg) Ehrenberg¹².

At Cordon (site 3) a riverbank exposure at the front of the same buried flat on which site 2 is located shows a similar stratigraphy to that at site 1 (ref. 1). At both transitions the appearance of *Compositae*, *Chenopodiaceae*, *Plantago* sp., *Filipendula*, *Rosaceae*, *Typha angustifolia* and *Lemnaceae* may indicate a succession of salt-marsh and freshwater communities as suggested in the Forth valley¹¹. Marine and brackish water diatoms are prevalent within the basal carse assemblage with *Paralia sulcata* and *Rhaphoneis surirella* averaging over 50%. The basal peat date (3a, Fig. 2; altitude 2.8 m OD) is $8,370 \pm 45$ yr BP (SRR-1147), suggesting the removal of estuarine conditions slightly later than at site 2. This indicates abandonment of the frontal edge of the estuarine flat at a measurably later date than that of the shoreline 1 km to landward. The reappearance of estuarine conditions and initiation of peat burial (3b, Fig. 2; present altitude 3.2 m OD) occurred about $7,525 \pm 50$ yr BP (SRR-1394).

The basal peat dates at sites 1–3 all relate closely to the abandonment of two now-buried estuarine flats, the older and higher of which is the Carey flat. Three distinct buried steps occur above the Carey feature, and the rather thin peat on two of them has been dated at sites 4 and 5 (Fig. 1, Table 1). These higher buried steps are necessarily older than the lower ones already considered, yet the basal peat dates are much younger than at sites 1–3, and in fact differ little from the ages of the topmost peat layer at the latter sites. This suggests a lengthy

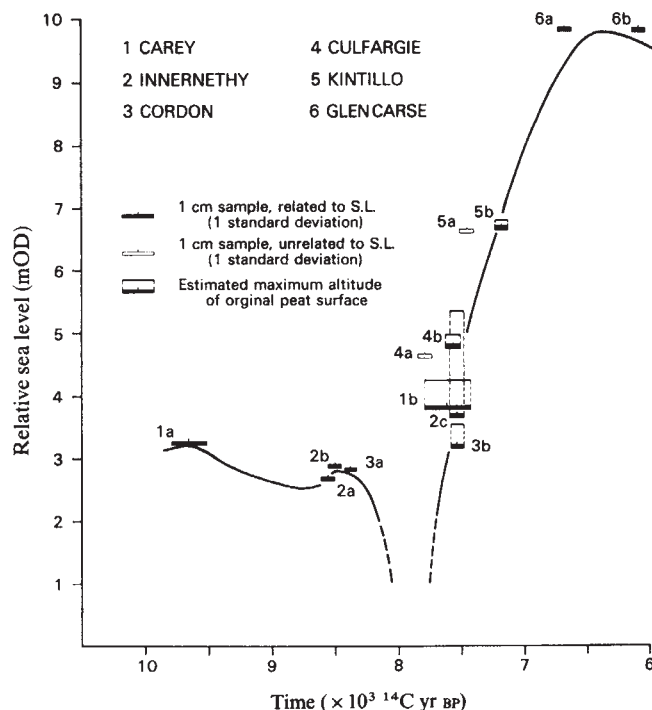


Fig. 2 Early Flandrian relative sea-level changes in the Carey-Cordon area, Lower Strathclyde. Horizontal bars extend one standard deviation either side of the ^{14}C dates.

hiatus between abandonment of the flats and the start of peat growth, and that the latter was a response to the rising ground-water level associated with the Main Postglacial transgression. The basal peat dates (4a and 5a, Fig. 2), although associated with marine transgression, cannot therefore be related to specific relative sea levels, and the higher buried flats remain undated.

Thin compacted peat at Culfargie (site 4) and Kintillo (site 5), 19 cm and 8 cm thick respectively, rests on fine sand and is overlain by 1.6 m and 3.5 m respectively of carse silty clay. Increased evidence of aquatic taxa is evident in the carse deposits at both sites and at Kintillo a sequence of freshwater and salt-marsh communities is suggested similar to that found at Cordon. Marine influence in the carse is also indicated by the abundance of *Paralia sulcata*. The basal peat dates at Culfargie

Table 1 Summary of details of radiocarbon-dated sites in Lower Strathclyde

Point no.	Site	Nat. grid ref.	Sample location	Altitude (m OD)	^{14}C age (yr BP) (uncorrected)	Lab. ref.
1a	Carey*	NO 1717 1710	Base of buried peat	3.19	$9,640 \pm 140$	I-2796
1a		NO 1747 1703	Base of buried peat	3.19	$9,524 \pm 67\ddagger$	SRR-72
1b		NO 1717 1710	Top of buried peat	3.78	$7,605 \pm 180$	NPL-127
1b		NO 1747 1703	Top of buried peat	3.78	$7,778 \pm 55\ddagger$	SRR-71
2a	Innerneath†	NO 1899 1783	Thin peat layer in silty sand beneath buried peat	2.66	$8,555 \pm 60$	SRR-1399
2b		NO 1899 1783	Base of buried peat	2.84	$8,505 \pm 50$	SRR-1398
2c		NO 1899 1783	Top of buried peat	3.64	$7,530 \pm 50$	SRR-1397
3a	Cordon*	NO 1845 1813	Base of buried peat	2.80	$8,370 \pm 45$	SRR-1147
3b		NO 1845 1813	Top of buried peat	3.16	$7,525 \pm 50$	SRR-1394
4a	Culfargie*	NO 1625 1717	Base of buried peat	4.60	$7,780 \pm 50$	SRR-1396
4b		NO 1625 1717	Top of buried peat	4.79	$7,555 \pm 50$	SRR-1395
5a	Kintillo†	NO 1348 1766	Base of buried peat	6.60	$7,465 \pm 55$	SRR-1401
5b		NO 1348 1766	Top of buried peat	6.68	$7,180 \pm 55$	SRR-1400
6a	Glencarse†	NO 2022 2256	Top of buried peat	9.52	$6,679 \pm 40$	SRR-1150
6b		NO 2022 2256	Base of surface peat	9.82	$6,083 \pm 40$	SRR-1151

* Riverbank exposure.

† Temporary excavation.

‡ These dates are not plotted in Fig. 2 because they were obtained on rather thick (4-cm) samples, and therefore relate less closely to the lithostratigraphic boundaries than the other dates, which were obtained from 1-cm thick samples.

(4a, Fig. 2; present altitude 4.6 m OD) and Kintillo (5a, present altitude 6.6 m OD), of $7,780 \pm 50$ yr BP (SRR-1396) and $7,465 \pm 55$ yr BP (SRR-1401) respectively, are verified by the high values for *Corylus/Myrica* pollen. Transgression of the peat surface occurred around $7,555 \pm 50$ yr BP (SRR-1395) at Culfargie (4b, Fig. 2; present altitude 4.8 m OD) and $7,180 \pm 55$ yr BP (SRR-1400) at Kintillo (5b, Fig. 2; present altitude 6.7 m OD).

In using peat-top dates to construct a relative sea-level curve account must be taken of the peat compaction that accompanied and followed burial by the coarse deposits (compaction of the sandy sub-peat materials is assumed to have been negligible). Peat compaction at sites 1, 2 and 4 was estimated by comparing the mean dry bulk density value for an uncompacted monocotyledonous peat at Hole of Clie (NO 2034 2291), in the Carse of Gowrie, with similarly derived values for the buried peats in Lower Strathearn, on the assumption that the fossil peats, before burial, had a comparable bulk density to the surface peat, and that the amount of subsequent compaction is directly proportional to the increase in bulk density. To allow for variations in bulk density within the peat, the mean value for each site was derived from measurements carried out at vertical intervals of 1–4 cm through the peat. The estimated values for compaction at sites 1, 2 and 4 range from 40 to 68%, and in the absence of direct measurements at sites 3 and 5, an overall mean value of 52% was used at those sites. The estimated original altitudes of the peat surfaces at the time they were transgressed by the sea are shown in Fig. 2.

The age and altitude of the Main Postglacial Shoreline, marking the culmination of a major transgression, have been measured at Glencarse (site 6), where a 30 cm-thick layer of coarse deposits is underlain and overlain by peat. The buried peat was transgressed (6a, Fig. 2) about $6,679 \pm 40$ yr BP (SRR-1150), and peat growth on the abandoned surface of the coarse deposits (6b; altitude 9.8 m OD) was under way at $6,083 \pm 40$ yr BP (SRR-1151)⁷.

Two characteristics of the relative sea-level curve (Fig. 2) as yet unexplained are the transgressive nature depicted for the Carey buried shoreline (point 1a) and the fall of relative sea level between points 3a and 3b. The former is in line with the transgressive nature suggested for the correlative Main Buried Shoreline in the Forth area^{6,14}. The latter is suggested by a buried channel excavated to a few metres below OD near the present River Earn^{11,15}. This low point on the curve occurs between $8,370 \pm 45$ and $7,525 \pm 50$ yr BP, whereas the equivalent trough in the Forth curve is dated to between $8,690 \pm 140$ and $8,270 \pm 160$ yr BP (ref. 4). However, the discrepancy may be negligible when account is taken of the wider timespan delimited for this event by the available Lower Strathearn dates, and the possibility that the low relative sea level occupied a very brief timespan after $8,370 \pm 45$ yr BP.

The relative sea-level curve (Fig. 2) is primarily the net result of isostatic land uplift and eustatic sea level changes. A curve of land uplift (Fig. 3) for the period 9,500–6,500 yr BP was con-

structed by a similar method to that of Sissons and Brooks⁴. Land uplift at 500-yr intervals was calculated by adding the amount by which world sea level was below the present level at each date, according to a particular published eustatic curve, to the contemporary relative sea level in Lower Strathearn as shown in Fig. 2, and subtracting 2 m to compensate for the fact that the data in Fig. 2 relate to former estuarine shorelines and therefore to high water mark. Curves were drawn for all eight eustatic curves^{16–23} not rejected as unsuitable according to the criteria used by Sissons and Brooks, and the results averaged to produce Fig. 3. The form of the curve is strikingly similar to the Forth uplift curve, the magnitude of uplift being less in Lower Strathearn because of the greater distance from the centre of uplift.

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Effect of aluminium speciation on fish in dilute acidified waters

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Acidification of lakes and streams is a serious water quality problem in high elevation granitic ecosystems in the northeastern US^{1–3}. An important consequence of acidification is the mobilisation of aluminium from the edaphic to the aquatic environment^{3–5}. Elevated levels of aluminium may have serious ramifications for biological communities, particularly fish, inhabiting acidified aquatic systems^{5,6}. In this study, water quality data were collected from several acidified lakes and streams in the Adirondack region of New York state. The purpose of this investigation was to characterise aluminium chemistry in these acidified waters and to assess the relative toxicity of soluble aluminium species to fish. Aqueous aluminium speciation was found to be highly variable in Adirondack waters, and its effect on fish was also variable.

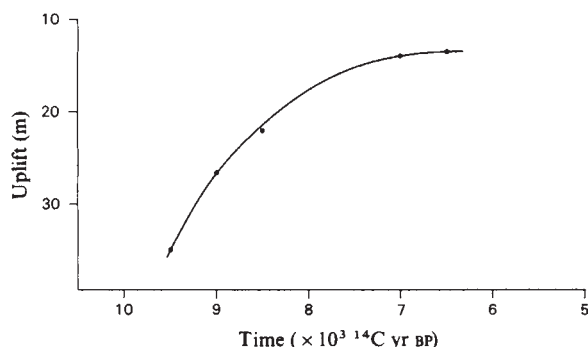


Fig. 3 Land uplift in the Carey-Cordon area between 9,500 and 6,500 yr BP. The graph shows total land uplift between each date and the present.

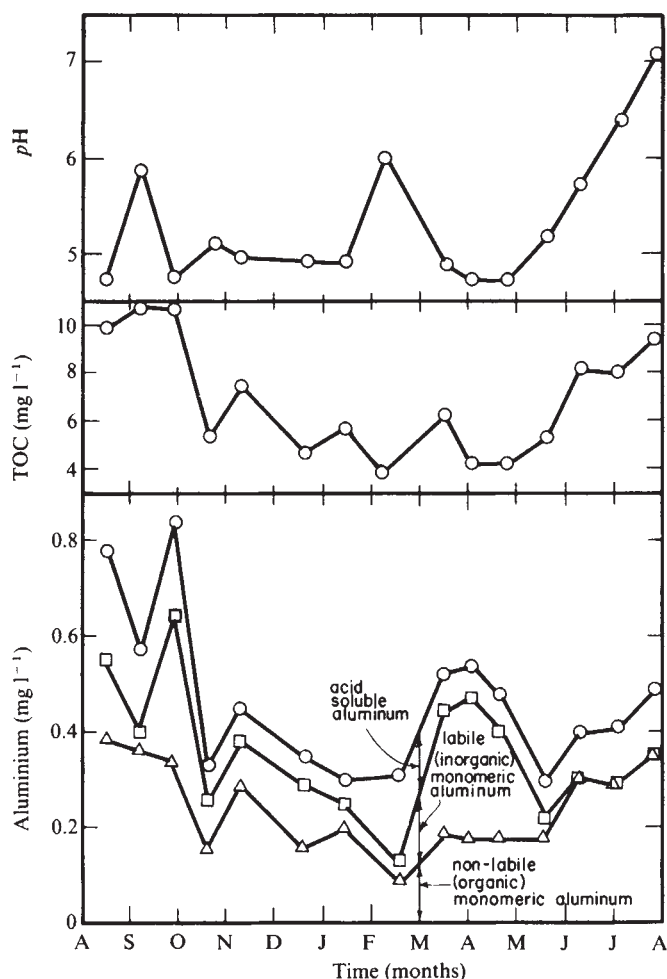


Fig. 1 Temporal changes in pH, total organic carbon (TOC) and aluminium forms for Canachagala Creek from August 1977 to August 1978. Low pH and high levels of labile monomeric (inorganic) aluminium were observed after rainfall (15 August, 27 September) and during snowmelt (15 March, 2 April and 23 April). Low water flow conditions produced elevated pH values and decreased labile monomeric (inorganic) aluminium levels. TOC and non-labile (organic) aluminium were generally high in the autumn and summer and low in the winter and spring. ○, Total aluminium; □, total monomeric aluminium; △, non-labile (organic) monomeric aluminium.

Three lakes in the southwestern part of the Adirondack Mountain Region were investigated—North Lake (74°55' W, 43°43' N; elevation 545 m); Big Moose Lake (74°50' W, 43°30' N; elevation 556 m); and Little Moose Lake (74°55' W, 43°42' N; elevation 545 m) and their tributaries were sampled over the course of a year. The edaphic, climatic and physiographical features of the Adirondack Region have been described by Schofield¹.

Three separate aluminium measurements were made on water samples using a colorimetric (feron) method⁷. Measurements included total aluminium (samples acid digested before analysis), total monomeric aluminium (samples analysed without acid digestion) and non-labile monomeric aluminium (aluminium separated by a cation exchange chromatography technique and analysed as monomeric aluminium). From these measurements, the total aluminium content of each sample could be subdivided into three fractions. Non-labile (organic) monomeric aluminium was measured directly and included organically chelated aluminium. The second fraction, labile (inorganic) monomeric aluminium, was determined as the difference between total monomeric aluminium and non-labile (organic) monomeric aluminium. This fraction includes free

aluminium and aqueous inorganic complexes (fluoride, hydroxide and sulphate). The third fraction, acid soluble aluminium, was determined as total aluminium minus total monomeric aluminium and includes aluminium forms that require acid digestion before analysis (polymeric, colloidal, extremely stable organic and hydroxy organic complexes). pH was measured potentiometrically; fluoride was measured potentiometrically with a fluoride-selective ion electrode; sulphate was measured with the turbidimetric method and total organic carbon was measured by sample oxidation and IR detection of carbon dioxide using an organic carbon analyser. The labile inorganic fraction of monomeric aluminium was subfractionated using thermodynamic calculations into free (aquo) aluminium and fluoride, sulphate and hydroxide complexes of aluminium. Details of the analytical procedures and thermodynamic calculations used here are given by Driscoll⁸.

Some typical temporal changes in measured aluminium, pH and total organic carbon for Canachagala Creek, a tributary of North Lake, are given in Fig. 1. The relatively high concentrations of aluminium are representative of those found at the other sampling sites.

Table 1 Estimated 50% survival times of brook trout (*Salvelinus fontinalis*, Mitchell) in synthetic soft water solutions containing aluminium and aluminium complexing ligands

pH	Ligands added	Total aluminium concentration mg l ⁻¹	% Survival after 14 days		Estimated 50% survival time (h)	
			Mean	Range	Mean	95% Confidence limits
5.2	—	0.02	99	95–100	>500	—
	—	0.42	28	20–30	115	88–150
	Fluoride	0.50	45	35–45	202	136–302
	Citrate	~0.5	96	87–100	>500	—
4.4	—	0.01	95	80–100	>500	—
	—	0.48	42	27–53	256	213–306
	Fluoride	0.47	71	67–80	472	354–629
	Citrate	~0.5	82	40–100	>500	—

50% survival times are maximum likelihood estimates based on a logistic model. Experiments were run for 14 days (336 h). Estimated survival times greater than the length of the experiment are based on extensions of the non-linear regression and are subject to large uncertainties. Thermodynamic calculations indicate that levels of ligands added should chelate nearly all aluminium present. Fluoride was added as NaF at 0.5 mg l⁻¹ F; citrate at Na₃H₅O₄ at 11.7 mg l⁻¹ C, at pH 4.4. At pH 4.4, all solutions were adjusted for equivalent Na levels (9.1 mg per l Na). The presence of citrate interferes with the measurement of aluminium. Aluminium levels are based on subsamples of water within experimental units. Each treatment had four replicates with 15–20 fish per replicate.

Periods of low pH were observed in the late summer and autumn after rainfall and during spring snowmelt. During the summer (1978) conditions were dry and pH levels consequently increased. During low pH conditions, total aluminium levels increased dramatically. However, the two fractions of monomeric aluminium (inorganic and organic) responded differently to changes in hydrogen ion activity. Increased levels of labile (inorganic) aluminium (Al³⁺ and aluminium fluoride, hydroxide and sulphate complexes) accounted for most of the response to decreasing stream pH and increasing flow. During high pH conditions, the inorganic fraction of aluminium was substantially reduced.

Variations in organically chelated aluminium seem to be independent of pH but are significantly correlated with total organic carbon measurements ($P < 0.0001$). In the tributaries studied, total organic carbon and non-labile (organically chelated) aluminium levels were low in the winter and spring and high in the summer and autumn. Therefore, the large fluctua-

tions in total aluminium concentration with pH in streams may be largely attributed to large fluctuations in the labile (inorganic) fraction of aluminium.

Aluminium forms strong complexes with hydroxide, fluoride, sulphate and dissolved organic ligands⁹⁻¹¹. All these potential aluminium ligands are present in the dilute acidified waters of the study lakes and streams. Results indicate that non-labile (organic) aluminium is generally the predominant fraction of aluminium in these waters. Thermodynamic computations indicate that fluoride complexes are generally the dominant inorganic aluminium species. Free (aquo) aluminium and aluminium hydroxide complexes were predicted to be present in lesser amounts and aluminium sulphate complexes were generally insignificant.

Elevated levels of aluminium in acidified surface waters may have serious effects on fish and other aquatic biota. Schofield and Trojnar⁶ indicated that levels of aluminium may be a primary factor limiting survival of trout stocked into acidified Adirondack lakes. However, speciation of aluminium was not considered in their study or in other studies of aluminium toxicity^{5,12}. It is generally accepted that complexation alters both distribution and toxicity of trace metals^{13,14}. Laboratory experiments from this study suggest that such conclusions are also applicable for aluminium.

Brook trout fry (*Salvelinus fontinalis*, Mitchill) were exposed in soft water to aluminium as the free ion and complexed with hydroxide, fluoride and/or citrate (Table 1). Fry were held in 8-l polyethylene units (20 fry at pH 5.2, 15 fry at pH 4.4 per unit, mean length 22–23 mm) with stock solutions changing at a rate of ~8 l per day. Stock solutions were prepared at least one day before use. Experimental units were aerated and temperatures ranged from 9 to 17 °C. Soft water was prepared with a Culligan reverse osmosis system using Ithaca city tap water and dechlorinated with a Barnstead organic removal filter. Basic cation concentrations in the softened water were 2–4 mg l⁻¹ Ca, 0.4–0.7 mg l⁻¹ Mg, 1.2–1.5 mg l⁻¹ Na and 0.2–0.4 mg l⁻¹ K. Total fluoride levels were less than 0.01 mg l⁻¹ F; sulphate levels were less than 5 mg l⁻¹ SO₄. Solutions were acidified with hydrochloric acid.

Treatments with excess fluoride or citrate decreased the toxicity of aluminium solutions (Table 1). Survival of brook trout fry after 14 days in treatment waters with 0.5 mg l⁻¹ Al and 11.7 mg l⁻¹ C citrate was 87% or greater at pH 5.2 and 40% or greater at pH 4.4, and not significantly different from control solutions at equivalent pH values. Additions of fluoride to acidified aluminium solutions also increased survival ($P < 0.001$ at pH 5.2, $P < 0.10$ at pH 4.4, Table 1). However, the mitigating effect of fluoride complexation on aluminium toxicity was less than that of organic complexation. Inorganic aluminium forms, therefore, seem to be the major species of concern with regard to aluminium toxicity in fish. As a result of reactions with hydroxide, aluminium toxicity to fish is pH-dependent. Schofield and Trojnar⁶ report increasing brook trout mortality and gill damage with increasing pH over the range 4.4–5.2 at fixed total aluminium levels (0.2–0.5 mg l⁻¹ Al).

Evaluation of aluminium speciation in acidified Adirondack waters indicates that organically complexed (non-labile monomeric) aluminium is the dominant form (Fig. 1). As complexation of aluminium with organic ligands seems to eliminate its toxicity, measurement of total aluminium concentrations may lead to a substantial overestimate of the potential aluminium-induced toxicity of acidified Adirondack waters. A comparison of mortalities of white sucker fry (*Catostomus commersoni*, Lacepede) in natural waters and in synthetic acidified water solutions containing aluminium (without additional complexing agents) supports this conclusion (Fig. 2).

For experiments with white sucker fry, 5-l polyethylene containers with 20 fry per unit were used. Approximately 1.5 l of stock solution was added to each experimental unit four times per day. Units were aerated and temperatures ranged from 17 to 19 °C. Two separate experiments were carried out at pH 5.0: 15–20 July 1978, mean length of fry at start of experiment

14.0 mm; and 7–12 August 1978, mean length of fry 18.7 mm. The sensitivity of white sucker fry to acidity and aluminium changes rapidly with age during the first few weeks after hatching (J. P. Baker, unpublished data). The fry become markedly less sensitive to low pH levels with time, but there may be a small increase in sensitivity to aluminium with increasing age.

Mortality rates of white sucker fry in three naturally acidified Adirondack waters (pH 5.0) appear to be determined by the levels of labile inorganic aluminium present (Fig. 2).

Water quality in dilute Adirondack waters is extremely variable, particularly with regard to organic carbon and hydrogen ion concentration. These variations have considerable effect on the aqueous aluminium chemistry, which in turn affects the survival of fish populations. Surges of labile (inorganic) aluminium into Adirondack streams during snowmelt and heavy rainfall are potentially lethal to eggs and fry. In addition, the ameliorating effect of organic complexation on aluminium toxicity suggests that lakes and streams with high organic carbon content may be suitable for successful fish production despite

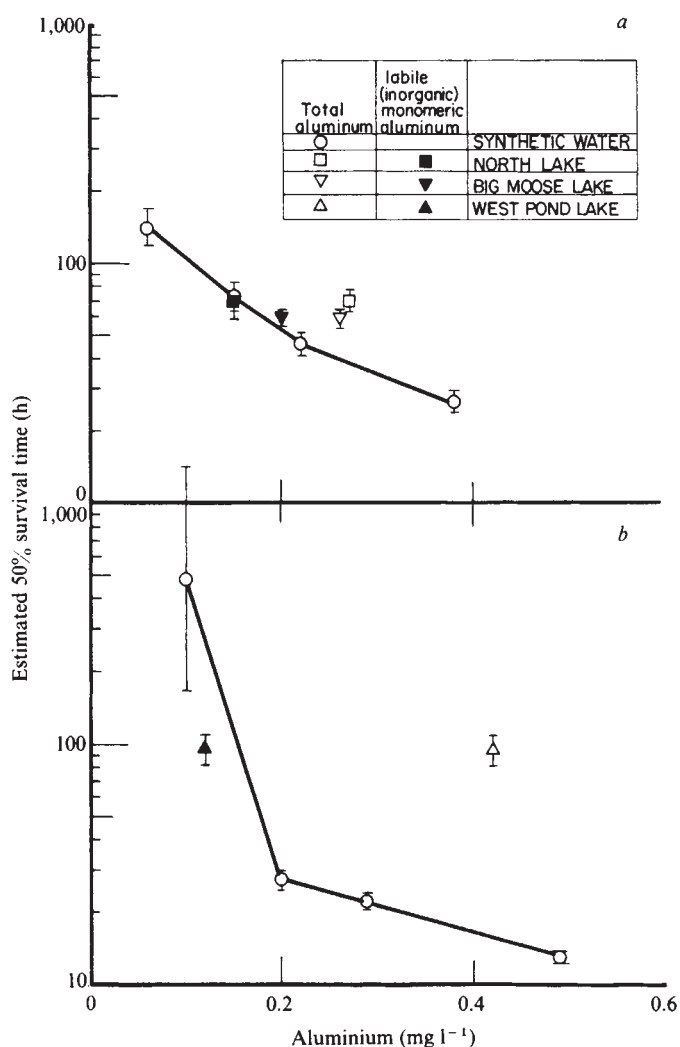


Fig. 2 Estimated 50% survival times (with 95% confidence intervals) of white sucker fry (*Catostomus commersoni*, Lacepede) at pH 5.0 in synthetic soft water solutions containing aluminium and in natural lake (Big Moose Lake, North Lake) and stream (West Pond Outlet, a tributary to Big Moose Lake) waters. 50% survival times are estimated as described in Table 1. Each value represents three or four replicate experiments with 18.7 mm fry with no aluminium added, survival was 95–100% after 5 days, and no 50% survival time was estimated. Mean fry length: a, 14.0 mm; b, 18.7 mm.

moderately low pH and high total aluminium levels. A detailed understanding of aluminium chemistry is essential to enable the effect of aluminium on the development of indigenous fish to be evaluated.

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Motion smear

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It is well known that the visual system summates signals over time, about 120 ms in daylight^{1,2}. Although this summation has the obvious advantage of enhancing visual sensitivity, it creates the potential problem of motion smear when viewing moving targets, whose images are also summated over time³. Here I report some measurements which reveal that provided the moving target is exposed for long enough to elicit a clear sensation of motion, the amount of smear is far less than may be expected. This suggests that the visual mechanisms which signal motion are also responsible for signalling a clear unsmear perception of the target in motion.

In addition to the threshold measurements of temporal summation it is also well established that super-threshold stimulation leads to a sensation which persists in vision for some time after the termination of the stimulus⁴, again, for about 120 ms in daylight^{5,6}. Perhaps the most direct measurements of the duration of this persistence were made by Ross and Hogben⁷ with dynamic random dot patterns, continuous sequences of brief flashes of light displayed at random positions on an oscilloscope face. Although only one dot is physically imaged on the

retina at any one time, an observer sees not just one, but a whole screen full of dots, in fact, all those that were displayed during the previous 120 ms. Clearly, under these circumstances, the neural sensations greatly outlast the duration of the visual stimulus.

This fact is of little consequence when viewing a stationary scene, but for moving targets, it poses the problem of motion smear. Objects travelling at even moderate speed cover a perceptible distance in 120 ms. If the same rules of visible persistence apply for moving scenes as for random dot patterns, we may expect moving objects to seem smeared and elongated in the direction of motion. This is illustrated by the photograph of Fig. 1, which was taken with a 125-ms shutter speed. Although the buildings all have good definition and contrast, the images of the people walking in the foreground are smeared beyond recognition. However, in natural viewing conditions, the images of moving objects always appear sharp and clearly defined, certainly very different from Fig. 1. Only very rapid image motion creates perceptible smear. Admittedly, with free viewing, the eyes will pursue a target of interest, but this does not solve the general problem, as it in turn introduces retinal motion of the background. What, then, are the mechanisms of vision that prevent motion smear?

Although this problem is fundamental to the understanding of dynamic visual perception, it has received surprisingly little attention. Many measurements have been made on the effect of image motion on acuity^{8,9}, but these do not necessarily reflect the more subjective perceptual qualities of smear or apparent elongation. Two investigations which do bear on this question report that the clarity and veridical perception of a target in



Fig 1 Vision through a 125-ms temporal window. Although much evidence suggests that the visual system integrates light for 125 ms, our every day perceptions differ markedly from a photograph taken with this integration period (shutter speed).

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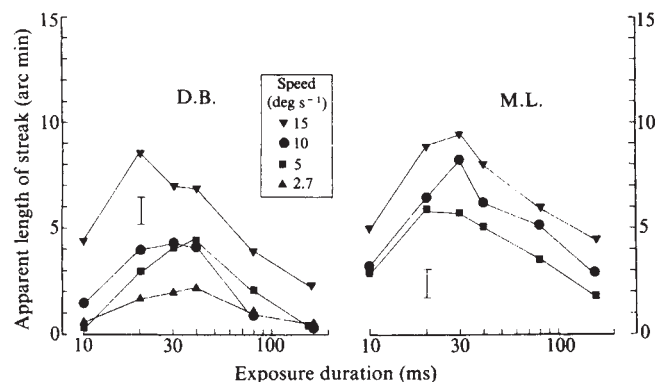


Fig. 2 The apparent length of the motion streak of dots moving along a constant linear trajectory, as a function of exposure duration. For durations of up to ~30 ms, the apparent length increases with increasing duration, as may be expected were light to be integrated over this period. However, at durations of longer than 30 ms, the apparent length decreases with increasing durations—the further the dots travel, the shorter they appear. The error bars each represent 2 standard errors, averaged over all conditions.

motion are enhanced by the prior and post exposure in locations at either end of the motion sequence^{10,11}. Here I show that even without pre- and post-viewing of stationary targets (which, given the filtering properties of vision, alters the effective visual velocity¹²), the amount of apparent elongation or smear is far less than might be expected on the basis of visual persistence⁷.

With a simple matching technique, I have measured motion smear as a function of the duration of exposure to the motion sequence. The measurements were made by matching the length of a short stationary line to the apparent length of dots in an array of motion, using a multiple interleaved staircase procedure under computer supervision¹³. The observer saw a short sequence of moving dots, immediately followed by a continuously visible stationary line. By pressing the appropriate button, he indicated which of the two stimuli seemed to be longer. Depending on his response, the length of the line was either incremented or decremented on the next trial of that condition. The apparent length, taken as the mean of the previous 15 trials, was measured separately three times to give a rough estimate of measurement error. The stimulus pattern was an array of 100 dots in random locations, intensified on an oscilloscope face (p15 phosphor) to a contrast 100 times their visibility threshold against a uniform background luminance of 30 cd m⁻². The dots were caused to move by displacing them successively at 5-ms intervals between each brief (4 μ s) intensification, on some trials to the left and on some to the right, the two directions being presented in random order. Short presentations of unpredictable direction of motion precluded the possibility of pursuit eye movements¹⁴. The screen was vignetted down to a 5° diameter circle by a mask of the same mean luminance placed outside the observer's field of focus, so that the dots faded smoothly out of view. Measurements are reported for two observers, D.B. and M.L., but the effect has been verified qualitatively with many observers, and indeed, can be readily verified by anyone with access to basic visual display facilities.

The results are shown in Fig. 2. Dots exposed for long durations, which travel over a larger retinal region, may be expected to seem more elongated than those exposed only briefly. Indeed, if all the light impinging on the retina were seen simultaneously, as with the dynamic random dot patterns, the perceived length of the streak should be equal to the distance covered by the moving dot. However, this is far from being the case. At short durations (up to 20–30 ms) the amount of motion smear does increase with duration, but at longer durations, the perceived length actually decreases with duration—the further the dots travel, the shorter they seem. Whereas with brief presentation times one sees an array of almost stationary line segments, at longer durations the stimuli are unquestionably small dots in motion, with no hint of elongation along their

trajectory, except when moving at the fastest speed, 15 deg s⁻¹. However, even at this speed the apparent length was only 2 or 4 arc min (for D.B. and M.L.), compared with the 100 arc min the spot traverses during the 120 ms of 'visual persistence'.

Not only was there less elongation of the dots, but also the sensation of motion became much stronger at longer durations. All the stimuli seemed to move to some extent, but with brief exposure times the sensation was quite weak. The non-occurrence of motion smear seems to be linked to the perception of smooth motion. Only when the motion seemed smooth and realistic was the target seen as a small, sharp, unsmearred dot. This suggests, that, contrary to the currently favoured idea of separate analysis of pattern and motion^{15,16}, the mechanisms of vision specialised to detect motion are also responsible for the analysis of the spatial form of the target in motion. Brief glimpses of a motion sequence fail to activate these mechanisms, activating instead mechanisms which, like a camera of long exposure, signal motion smear. A more detailed account, explaining these and other similar results in terms of the known spatial and temporal tuning properties of visual mechanisms, is in preparation.

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Mastectomy and mammary glands in reproductive control in the goat

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Little attention has been paid to the role of the mammary glands in reproduction apart from the suppression of reproductive activity during lactation in some species. A hint of greater involvement, however, stems from two observations in the goat. First, Linzell¹ reported that while developing his technique of transplanting mammary glands to other sites in the body in order to gain access to the mammary artery, the incidence of reproductive disturbances (infertility, abortion and apparently inexplicable maternal death at parturition) seemed greater in goats which had lost mammary tissue post-operatively, or which retained only one mammary gland, than in others in the herd and second, the mammary glands may significantly influence the concentration of a hormone in the general circulation²⁻⁵. Moreover, in some marsupials there is evidence that the mammary glands are involved in the control of reproduction, for example, denervation of the mammary glands during lactation initiates development of the diapausing embryo⁶. In view of the importance of reproductive integration in the artificial control of fertility, we have followed up these clues by investigating the effects of mastectomy in the goat, a species with a seasonal, rather than a lactational, anoestrus. We report here that the oestrous cycle was markedly disturbed by mastectomy and that fertility, and possibly also the length of gestation, seemed to be affected.

Table 1 Effects of mastectomy on oestrous cycles of goats approximately 1 yr after operation (s.e.).

	Length of oestrous cycle* (days)				Duration of behavioural oestrus (h)	
	1st	2nd	3rd	4th	1st	Subsequent
Intact controls (5)	22.0 ± 1.48	21.6 ± 0.25	21.4 ± 0.25		approx. 30	approx. 30
Operated controls (5)	24.4 ± 0.51	21.0 ± 0.01	21.6 ± 0.25		approx. 30	approx. 30
Mastectomised (5‡)	8.4 ± 0.40	13.0 ± 2.45	17.0 ± 1.41	14.3 ± 2.21	115 ± 13.9	approx. 30
	$P < 0.001$	$P < 0.01$	$P < 0.05§$			

Values given are means ± s.e. P values are with respect to both intact and operated groups except that marked §, $P < 0.01$ compared with the operated controls.

* First day behavioural oestrus observed = day 1 of cycle.

† Between first and second oestrus, and so on.

‡ One goat developed hepatitis during second cycle and had to be killed.

Both mammary glands were removed, under halothane anaesthesia, from five goats aged 9 months. The operations were done in early December (that is during the breeding season) 3–7 days after the last observed oestrus. No sham operation could be devised to mimic closely the full procedure of mastectomy. Therefore, the operated animals were compared with two control groups—five intact goats and five which had been subjected to udder surgery (such as ligation of blood vessels crossing between the two glands⁷) akin to that completed by mastectomy but in which neither the main nerves nor major blood vessels had been divided. Although in young virgin goats mastectomy is a relatively minor surgical procedure, the results obtained in the first breeding season are not presented to avoid the possibility that the effects observed might be attributed to nonspecific effects of surgery. However, it is interesting that one goat never returned to oestrus for mating and in two others fertility seemed to be reduced.

In the second breeding season, approximately 1 yr after operation, the goats were observed several times each day for the signs of behavioural oestrus (bleating, restlessness and tail-wagging). The mastectomised goats showed the first signs of behavioural oestrus during a period in the autumn indistinguishable from that of other goats in the herd; in other words they emerged from seasonal anoestrus at the same time. However, major differences then became apparent. Thus the first oestrus of the season lasted 3–6 days in the mastectomised goats compared with approximately 30 h in the controls. Moreover, the first cycle (between the onset of the first and the second oestrus) was significantly shorter in the mastectomised animals (mean 8.4 days) than in either the intact (22 days) or operated (24.4 days) controls. Subsequent cycles were also significantly shorter, although the duration of behavioural oestrus was not apparently affected (Table 1). The mastectomised goats were all mated at the second oestrus but all returned to oestrus at least twice more (two, two, three and four times, respectively, in the four surviving goats (Table 1), even though they were mated each time. By contrast, the controls that were mated first (with the same males) at the fourth oestrus showed no further cycles. Moreover, in the rest of the herd mated at the first to fourth oestrus of the season, only four out of 80 matings (in two seasons) did not result in conception at the first attempt.

The gestation period (in days) of the four goats that became pregnant in their first season (number of young delivered in parentheses) were: 146 (2), 146 (1), 146 (1) and 147 (1), and in the second season: 143 (2), 147 (1), 147 (2) and 148 (2). Because the number of young carried seems to affect gestation period, comparison with controls is difficult. Nevertheless, for those bearing one kid the gestation periods were all shorter (by a median of 4.5 days) than the median of 151 days for goats in the herd⁸; similarly, for those bearing two the gestation periods were all shorter (by a median of 3.5 days) than the median of 150 days⁸. All young were delivered alive.

These results indicate that mastectomy had a pronounced effect on oestrous cycles in the goat; in addition, fertility, and possibly also the duration of pregnancy, seemed to be affected. Further experiments are in progress, but it would seem that

there is an alteration in the 'fine-tuning' of reproductive processes. It can be argued that it is the presence of mammary tissue rather than lactation which is of major importance in the effects described because there seems to be no difference between lactating and non-lactating intact goats in terms of the periodicity of oestrous cycles or of fertility (our unpublished observations).

Although Linzell¹ found no apparent effects of mastectomy on reproduction in the guinea pig, the strategies of reproductive control used by mammals are so diverse that it remains to be seen whether reproductive difficulties supervene in individuals of other species which suffer loss of mammary glands or tissue, either surgically as in women with mammary tumours, or following infection as in mastitis in farm animals.

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Evidence for selection following perturbation of allozyme frequencies in a natural population of *Drosophila*

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There is now ample evidence for the ubiquity of genetic variation at allozyme loci in many species^{1,2}, but the extent to which this variation is actively maintained by natural selection remains unresolved. Although various approaches have suggested the importance of selection at some allozyme loci in laboratory populations^{3–6}, to be fully convincing, evidence for natural selection must finally come from studies on natural populations. Inevitably, this will entail an understanding of and the potential to manipulate both the ecology and genetics of the population. Because sufficient is known of the ecology, breeding site, nutritional requirements and field behaviour of at least some species of the cactophilic *Drosophila*, we^{7–9} and others¹⁰ have chosen them for experimental evaluation of the forces operating to maintain genetic variation. If genetic variation at some locus is being actively maintained by natural selection, the action of this selection should be detectable following artificial change in gene frequencies produced either by adding certain genotypes to the population, or by removing them from it. Jones and Parkin¹¹ have reported unsuccessful attempts to detect selection by such

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perturbation experiments in *Cepaea* and *Drosophila pseudoobscura*, while Halkka *et al.*¹² inferred strong natural selection at a locus controlling colour polymorphism from exchange of individuals of *Philaenus spumarius* between two island populations. We report here results for the simultaneous perturbation of gene frequencies at three allozyme loci in *Drosophila buzzatii*. The changes in gene frequency following this perturbation provide strong evidence for selection, and for differential selection among the three loci.

The loci chosen for perturbation were *esterase-2* (*Est-2*), *pyranosidase* (*Pyr*) and *alcohol dehydrogenase-1* (*Adh-1*), which are on different chromosomes. The only known chromosome inversions are on the second chromosome¹³, and although *Est-2* is also on this chromosome, its precise relationship to the inversion has not been determined. The alleles whose frequencies were increased were the comparatively rare *Est-2*^c and *Pyr*^b, and the intermediate frequency *Adh-1*^c. At one population in the Hunter Valley, NSW, Australia (locality 5 of ref. 7), wild flies have been collected monthly over a 4-yr period to study temporal changes in gene frequencies. From wild females collected during this temporal study, 37 isofemale lines were made simultaneously homozygous for *Est-2*^c, *Pyr*^b and *Adh-1*^c. These lines were crossed in pairs, then double-crossed and progeny of the latter used to initiate two duplicate population cages.

The population that was perturbed inhabits an isolated clump of *Opuntia inermis* comprising about 40 large plants in an area about 50 × 20 m, and is located some 7 km from the temporal study site. The surrounding area is cultivated or open grazing land, and the nearest *O. inermis* is some 3 km distant. Four estimates of gene frequencies in this population (times 1–4, Fig. 1) were made over 8 weeks before perturbation began. The first

Table 1 Post-perturbation phase weighted average migration rates (*m*), range of estimated migration rates over the three loci and heterogeneity χ^2 for different periods

Times	<i>m</i>	Range	χ^2	
18–23	0.827	0.443–0.941	6.671	<i>P</i> < 0.05
18–24	0.951	0.742–1.042	0.898	
18–25	0.883	0.841–1.328	5.412	<i>P</i> < 0.10
18–26	0.990	0.821–1.217	9.162	<i>P</i> < 0.05
18–27	0.855	0.794–1.190	3.867	

release was done at time 4 after collection of flies for gene frequency estimation.

Perturbation was done in two ways. By continuous egg sampling from the homozygous laboratory cages, replicate cages were produced containing all developmental stages from eggs to newly enclosed adults. Two of these were set up in the field every 2–3 weeks, protected against predatory mice and insects and from the direct effects of sun and rain, but with an opening in the lid allowing eclosing adults to escape. Adults entered the population from these cages over a period of 3–6 weeks, depending on the season. In addition, we exploited knowledge of the breeding site of the species. It was thought that, by allowing the immature stages to feed and develop in their natural environment, they might be better adapted to enter the natural population than cage-reared adults. Accordingly, each time that cages were put out in the field, a thick suspension of eggs and young larvae from one to four of the most recently added cage cups was injected into as many rotting cladodes as could be located.

Thirteen such releases were made over 253 d (at times 4–17, excluding time 10). Gene frequency increased significantly at all three loci, but the pattern of change differed for *Est-2*^c and *Pyr*^b compared with *Adh-1*^c. The former initially increased rapidly, remained approximately constant for about 100 d during autumn–winter, and increased again in spring. In contrast, *Adh-1*^c increased steadily through autumn–winter. Selective effects may be implicated as *Adh-1*^c tends to be at higher frequency in the southern part of the species distribution⁷, and its frequency increased among survivors in cold shock experiments (A. W. Watt, personal communication).

After time 18, gene frequencies decreased to approximately pre-perturbation values, but again the patterns of change differed for *Adh-1*^c compared with *Est-2*^c and *Pyr*^b. The latter decreased steadily, but *Adh-1*^c showed a slower and more irregular decrease.

The observed increases in gene frequency during the perturbation phase must have been due to migration into the population of release flies, whereas the decreases in the post-perturbation phase could have been due only to selection or to migration from other populations. For any given time period, if changes in gene frequency were due only to migration, the estimated migration rates for each locus should not be significantly different. However, if there were any differential selection among loci, this could be detected as significant heterogeneity among the estimated migration rates. For each of the perturbation and post-perturbation phases, these migration rates and χ^2 tests of heterogeneity have been estimated¹⁴. During perturbation, migrants were assumed to be release flies only, and the estimated migration rates were not heterogeneous. Weighted average rates for different periods were: pre-perturbation to time 18, 0.458; times 4–18, 0.473; times 5–18, 0.523; times 6–18, 0.375. Thus, some 40–50% of gametes entering the population came from release flies. For post-perturbation, gene frequencies in any migrants are unknown, but were assumed equal to the pre-perturbation weighted average frequencies. Gene frequencies in any migrants from nearby populations are unlikely to be very different; for example, average frequencies in the temporal study population were *Est-2*^c 0.113, *Pyr*^b 0.149, *Adh-1*^c 0.505, as compared with the pre-perturbation weighted average frequencies of 0.120, 0.152 and 0.462, respectively. For the post-perturbation phase, weighted average migration rates

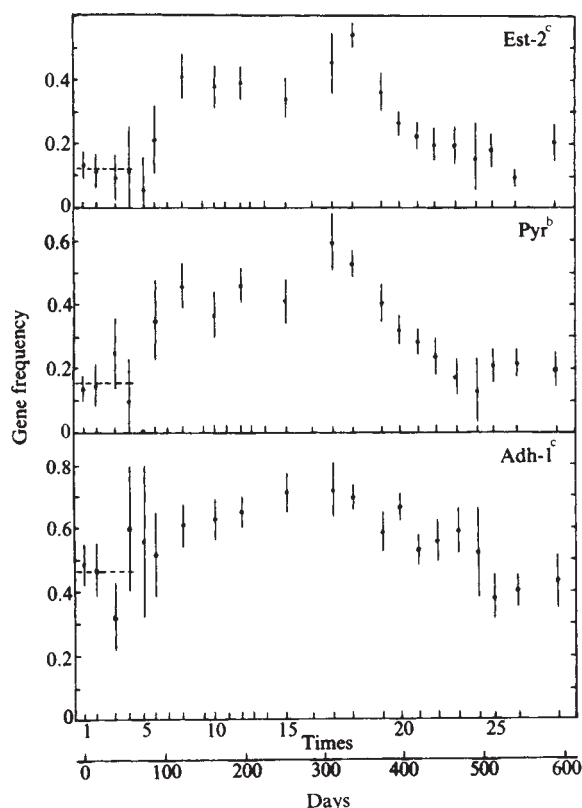


Fig. 1 Changes in gene frequency of *Est-2*^c, *Pyr*^b and *Adh-1*^c during perturbation during times 4–18, and following cessation of perturbation. The dotted line through times 1–4 is the pre-perturbation weighted average gene frequency. Times refer to the sequential number given to each collection and/or release. The gene frequencies plotted are those for non-release flies only, all collected flies that were homozygous *Est-2*^c, *Pyr*^b and *Adh-1*^c being excluded.

(m), the range of estimated migration rates for each locus and heterogeneity χ^2 for different periods were as shown in Table 1. Given the significant heterogeneity among migration rates and the extraordinarily high rates necessary to account for the changes in gene frequency, migration is not a sufficient explanation for the decreases in gene frequency. Therefore, the post-perturbation decrease of gene frequencies to the pre-perturbation values must be ascribed to natural selection.

We cannot argue from these data that selection has acted directly at the allozyme loci, and two arguments could be raised to suggest that selection may have been acting against chromosome regions marked by these loci. First, although 37 separate lines contributed to the release population, so that its genotype at other than the three loci and closely linked regions should reflect the variability in the natural population from which the lines derived, this latter population was not the one that was perturbed. However, as noted previously, it was only 7 km distant, average gene frequencies for polymorphic allozyme loci were very similar, and it was presumably subject to similar pressures of natural selection. Second, the wild females used to produce the homozygous lines were collected over 6 months, and the time from the middle of this period to the last release at time 17 was 21 months. Adaptation to laboratory conditions during this time may have been such that the post-perturbation effect was one of selection against laboratory-

adapted genomes. Although this is possible, it must be emphasised that during the perturbation phase these genomes were successfully incorporated into the wild population and at least partly recombined with wild genomes. Also, alleles favoured in the laboratory environment but deleterious in the wild should have been largely eliminated during this phase. Finally, there must have been differential selection among the three loci (or among chromosome regions marked by them) to account for the observed heterogeneity in estimated migration rates.

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Anaesthetics increase light emission from aequorin at constant ionised calcium

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The isolation by Shimomura, Johnson and Saiga¹ of a protein, aequorin, that emits light in the presence of micromolar concentrations of ionised calcium opened up new possibilities for the study of ionised calcium inside cells²⁻⁵. It is a relatively simple matter to introduce aequorin into large cells by microinjection, and the rate of light emission gives a direct measure of intracellular free calcium. During an investigation into the action of certain anaesthetics on squid axons, we noticed that these agents always increase the light output from intracellular aequorin. Subsequent analysis has now revealed that this does not result from a rise in ionised calcium inside the axon but seems to reflect a direct effect of the anaesthetic agent on the aequorin molecule. The agents studied all produce greater activation of the light emitting reaction at a constant level of ionised calcium. These rather simple observations have several important biological implications: (1) aequorin might be an interesting model system for studying interaction between anaesthetics and proteins, (2) other Ca-sensitive proteins might behave like aequorin, their affinity for calcium being subject to modulation, and (3) such modulation of endogenous Ca-binding proteins might contribute to the mechanism of anaesthesia.

Figure 1 shows the simultaneous measurement of aequorin light output and calcium efflux from a squid axon. Application of urethane produces a reversible increase in light output which is largely independent of the calcium content of the sea water. The most obvious explanation for this observation is that urethane has, in some way, raised the intracellular ionised calcium concentration, possibly by effecting release of bound calcium. The efflux data of Fig. 1 provide no evidence for such an effect because a rise in ionised calcium should lead to an increase in calcium efflux and the efflux does not change during exposure to urethane.

An alternative explanation consistent with Fig. 1 is that the anaesthetic is not altering intracellular ionised calcium but is

acting directly on the aequorin molecule. This possibility was investigated by introducing aequorin into a 500- μ m diameter porous cellulose acetate tube⁶ and superfusing the trapped aequorin with 0.5 M KCl solution containing 10 mM phosphate, pH 7.2, and a variety of Ca-EGTA buffers. Figure 2 shows that in this *in vitro* assay system urethane still increases light output even when ionised calcium is held constant by a relatively high concentration of calcium buffer. On a log-log plot the effect of urethane is to shift the relationship between light output and ionised calcium to the left without change in slope. As the total light output—assayed by injection of aequorin into a 0.5 M solution of calcium acetate—is not obviously different within the experimental error of about $\pm 5\%$ in the presence of up to

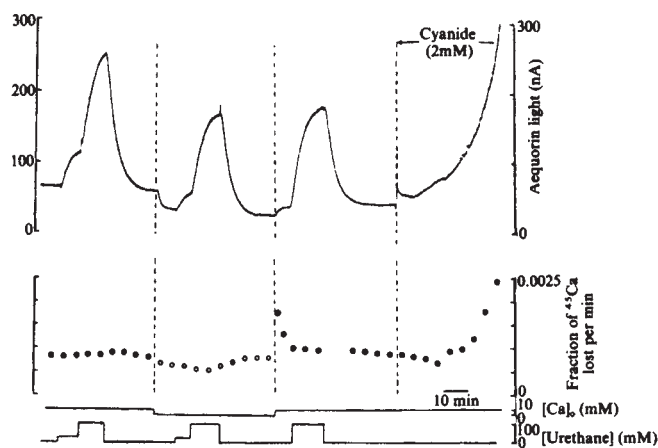


Fig. 1 Simultaneous measurement of aequorin light output (upper trace) and ^{45}Ca efflux (lower trace) during exposure of a cleaned axon of *Loligo forbesi* to urethane and cyanide. Axon 830 μ m; temperature 20 $^{\circ}\text{C}$. Axon immersed in artificial sea water containing (mM): NaCl, 400; MgCl_2 , 100; CaCl_2 , 10; KCl, 10; NaHCO_3 , 2.5. Calcium was removed for the period indicated. Urethane and cyanide were added to the sea water at the times shown. Note that during the period of urethane application, although the light output increased, calcium efflux was little affected whereas during application of cyanide both light emission and calcium efflux increased roughly in parallel.

200 mM urethane, these observations indicate that urethane is increasing the affinity of aequorin for calcium.

Urethane affects aequorin in the same concentration range as that required to produce anaesthesia. Half-maximal inhibition of the sodium current in squid axons requires about 100 mM urethane⁷. Clear effects of urethane on aequorin can be seen at 20 mM and the increase in rate of light emission is half-maximal also at about 100 mM. Essentially similar results have been obtained with several other anaesthetic agents (Fig. 3). Diethyl ether (100 mM), chloroform (50 mM), ethanol (100 mM), procaine (25 mM), tetracaine (2.5 mM) and althesin increase the light emission by aequorin at constant buffered concentrations of ionised calcium in the physiological range. However, our findings are at variance with those of Kamaya *et al.*⁸, who reported a reduction in calcium-induced light emission from aequorin exposed to a variety of anaesthetic agents. The reason for this difference is not clear. We have only seen inhibition of light output at very high concentrations of urethane and ethanol.

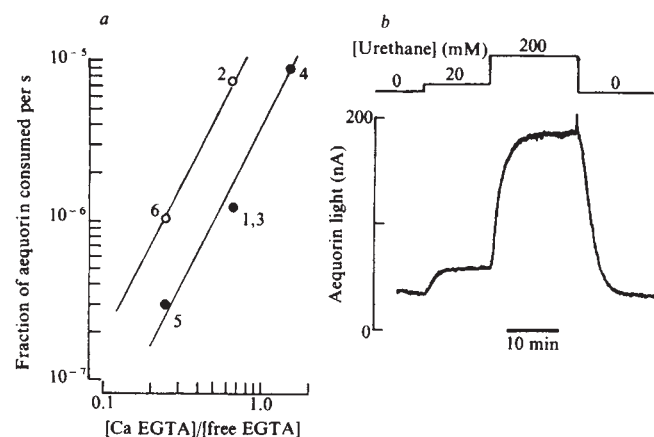


Fig. 2 Effect of urethane on aequorin light output *in vitro*. Continuous measurement of light output from a single sample of aequorin enclosed in a porous cellulose acetate capillary and superfused with test solutions of composition (mM): KCl, 500, potassium phosphate, pH 7.1, 20; Ca-EGTA buffer, 5. *a*, Shows a log-log plot of light output against ionised calcium in the absence (●) and presence (○) of 200 mM urethane. Points were obtained in the order indicated. *b*, Shows light output for a sample of aequorin exposed to 20 and 200 mM urethane in the presence of a Ca-EGTA buffer containing 4 Ca:10 EGTA. Temperature 20 °C.

These findings are of interest for two reasons. First, we have demonstrated a clear effect of a variety of anaesthetic agents on a well defined soluble protein; this raises once again the possibility that general anaesthesia may result from a direct interaction between anaesthetic and protein in the nervous system. Second, it is possible that other Ca-binding proteins behave like aequorin. It has long been recognised that many intracellular processes are regulated by Ca^{2+} ions. The first to be understood at the molecular level was the regulation of muscular contraction, where the binding of calcium to a low molecular weight protein, troponin C, produces a conformational change that leads to activation of myosin ATPase⁹. More recently, evidence has accumulated that troponin C is only one example of a whole class of Ca-binding proteins (calmodulins) that confer calcium sensitivity on intracellular processes¹⁰⁻¹². The characteristic features of these molecules are a relatively low molecular weight, more than one Ca-binding site and a large conformational change associated with uptake and release of calcium. The resemblance to aequorin is striking and it seems important to establish whether the affinity of these molecules for calcium may be subject to physiological and pharmacological modulation in a manner analogous to that which we have described for aequorin. This could add a further dimension to the already

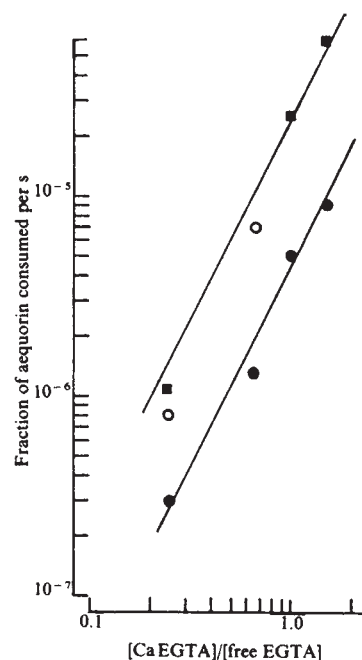


Fig. 3 Effects of diethyl ether (200 mM, ○) and tetracaine (2.5 mM, ■) on aequorin light output *in vitro*, and light output in the absence of anaesthetic (●). Data from two separate experiments carried out as described in Fig. 2*a* legend.

complex field of calcium regulation, because not only could reaction rates be altered by changes in free calcium, but they could also be controlled at constant ionised calcium by modifying the affinity of their respective binding sites (calmodulins) for calcium.

To return to the specific case of anaesthesia, anything that increases the permeability of nerve cell membranes to K^+ or Cl^- will render the cells less easy to excite. Both K^+ and Cl^- permeability systems exist in calcium-sensitive forms^{13,14} and Krnjevic¹⁵ has suggested that anaesthetics may activate these systems by producing a rise in intracellular free calcium. The present results suggest an alternative mechanism: anaesthetics may make these calcium-dependent permeability systems more sensitive to calcium, and so reduce excitability without the need for any change in intracellular ionised calcium. This may explain the recent observations of Simons¹⁶ that propranolol increases potassium permeability of human erythrocyte ghosts in which the internal calcium is stabilised by EGTA buffers. In addition to permeability effects, the other calcium-sensitive processes in nerve cells that merit close examination are transmitter release, Ca ATPase and the regulation of cyclic nucleotide levels.

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Potassium channels in nodal and internodal axonal membrane of mammalian myelinated fibres

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Horakova *et al.*¹ were the first to observe that the phase of late outward current carried by potassium ions in frog and squid nerve is virtually absent in voltage-clamped rat nodes of Ranvier. This observation has been recently confirmed by Chiu *et al.*² in rabbit nodes of Ranvier, suggesting that the nodal membrane in the mammal generally has few if any potassium channels. The present voltage-clamp experiments show that large potassium currents can, however, be produced in normal rabbit nodes of Ranvier by acute treatment designed to loosen the myelin from the axonal membrane. From this we conclude that whereas potassium channels are absent in the mammalian nodal membrane, they are normally present in the internodal axonal membrane at least in the paranodal region.

Figure 1Ai shows a series of ionic currents (after subtraction of a linear leakage current) obtained in a rabbit node of Ranvier. Starting at an initial holding potential of -80 mV the membrane was clamped to a series of equally spaced potentials in the range -72.5 mV to $+62.5$ mV. The early sodium current is clear in the records whereas there is very little late outward current. Such outward current as was present was quite insensitive to externally applied tetraethylammonium ion (TEA). Various procedures were then applied to loosen the myelin. Typically, the preparation was first exposed to collagenase (Sigma) for 2–10 min to loosen connective tissue. The solution bathing the node was then alternated between normal Locke solution and various modified Locke solutions designed to produce volume changes in the nerve. Three such solutions were used: a Locke solution in which half of the sodium was replaced by potassium, a hypertonic solution produced by adding 0.5 M sucrose to Locke, and a hypotonic solution obtained by diluting Locke with an equal amount of water. These procedures, undertaken in the hope of loosening the axonal membrane from the investing myelins, were repeated several times until a sudden and dramatic increase in the outward current was observed (Fig. 1Bi). Addition of tetrodotoxin (to block the sodium current) left only the outward current (Fig. 2A). External application of 20 mM TEA at this stage usually reversibly reduced the outward current by about 30%. However, when the solution in the end pools was changed from a potassium chloride solution to a caesium chloride (80 mM) and TEA chloride (80 mM) solution the late outward current decreased by about 70% in 10 min (Fig. 2C).

Concomitant with this large increase in outward current was a marked change in the shape of the transient capacity current. Whereas before treatment the initial capacity transient settled quickly in a fraction of a millisecond to a steady state leakage value (first few sampling points after the pulse in Fig. 1Aii), after treatment an additional slow current transient developed with a time constant of 0.5 – 1.5 ms (Fig. 1Bii). Experiments using hyperpolarising pulses to different potentials between -90 mV and -125 mV showed that this slow current was a linear capacity current. Thus, the time constant of this slow current measured during the pulse was voltage-independent; and it was about the same size as that measured after the end of the pulse. Furthermore, the time integral of this slow current (after subtraction of a steady leakage) obtained during the pulse (that is, the total charge displaced during the on-response) varied linearly with voltage, and roughly equalled that obtained in the off-response following the pulse. We believe that this slow capacity current

occurred because the 'myelin loosening' treatment had exposed a large amount of new axonal membrane previously covered by the myelin, and that the capacity of this newly exposed membrane was now being charged by the voltage-clamp through a large series resistance. For the particular node in Fig. 1, the

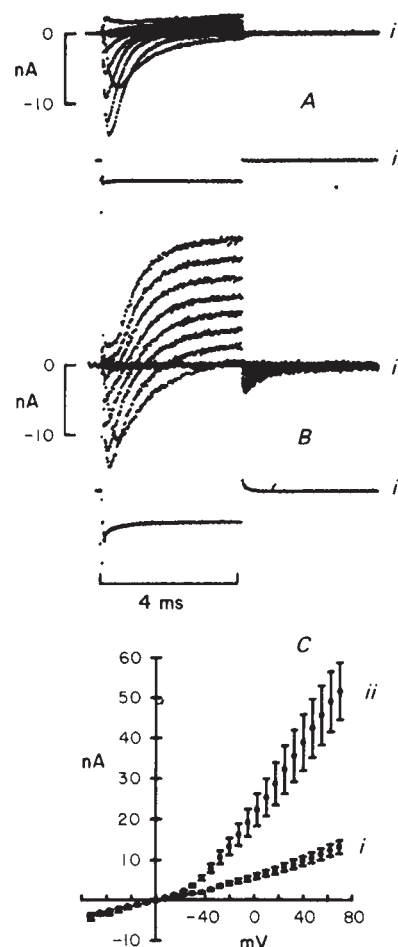


Fig. 1 Ionic currents in rabbit node before (Ai) and after (Bi) acute treatment to loosen the myelin. Families of current in both A and B were generated by the same series of depolarisations from an initial holding value of -80 mV to various test potentials in the range -72.5 mV to $+62.5$ mV in 15 mV increments. The currents were corrected for a linear leakage by appropriate scaling of the current response associated with a hyperpolarisation to -125 mV (Aii, Bii). The currents in A were measured in normal Locke after 30 min of repeated treatment (see text). During this period, the outward current and the nodal capacity showed practically no change. Currents in B were measured 3 min after A with the fibre in normal Locke when the late outward current suddenly showed a marked increase. Note that the capacity transient shows both a fast and a slow component; the latter is relatively small beforehand (Aii) whereas afterwards (Bii) it becomes much larger. The nodal capacity was obtained from records Aii and Bii by integrating numerically the capacity current during the pulse after correcting for a steady leakage. Since there were only a few computer sampling points during the fast capacity transient, the value for the capacity obtained by this procedure is based mainly on the slow component of current. The capacity per node obtained in this way was 2.5 pF in A and 35 pF in B. The steady leakage was 2.9 nA in A and 4.4 nA in B. Current signals were sampled every 30 μ s during the test pulse and every 150 μ s afterwards; and the time scale for the current after the pulse had been compressed fivefold compared to that used during the pulse. In addition, the fast capacity transients in A and B were sampled every 12 μ s. C shows the current-voltage relation of the total steady ionic current (mean values ± 1 standard error) for four fibres before (i) and after (ii) acute 'myelin-loosening' treatment (30–45 min). The steady currents were measured 4–8 ms after onset of the potential change. The holding potential was -80 mV for all experiments. The temperature was 24°C .

total capacity suddenly increased from an initial value of 2.5 pF to about 35 pF, with the increase coming mainly from the slow capacity current (see legend to Fig. 1). Interestingly, the dramatic increase in both the outward current and the capacity was not accompanied by a corresponding increase in the early sodium current (Fig. 1A,B), suggesting that the newly exposed axonal membrane has few sodium channels. Figure 1C shows the current-voltage relations of the total late current from four experiments in which both the increase in capacity and the outward current were sudden and particularly marked (the capacity increased by a factor of 20 ± 2.6 from an initial value of 3 ± 1.2 pF).

A simple explanation of these experiments is that the internodal axonal membrane, at least that in the paranodal region, normally contains potassium channels and the nodal axonal membrane does not. This idea is consistent with two recent experiments on mammalian nerve fibres in which the myelin was chronically disturbed by pathological means. First, Brismar³ has shown the consistent presence of potassium currents in alloxan diabetic rats; and it may be relevant that a characteristic of human diabetic neuropathy is a lifting of myelin away from the axonal membrane. Second, in rat dorsal root fibres exposed to diphtheria toxin, Sherratt, Bostock and Sears⁴ have shown that the outward membrane currents in regions of the nerve showing evidence of delayed saltatory or continuous conduction are sensitive to 4-aminopyridine and tetraethylammonium chloride, in contrast to their insensitivity at normal nodes of Ranvier as described by Bostock, Sherratt and Sears⁵. Finally, even in apparently normal nodes, potassium currents have occasionally been observed⁶, perhaps because the paranodal region was exposed by slight mechanical stretching during dissection.

The late outward current reported here is unlikely to be an artefact; and we believe that it is mainly a potassium current for the following reasons. First, it was blocked partially by external TEA and more effectively by allowing caesium and TEA to diffuse to the inside of the node. Second, the time constant of turn-on of this late current was voltage-dependent (as in Fig. 2D), and similar in size to the time constant for potassium

current observed previously in the frog. Thus as Fig. 2B shows the time course of the outward current in Fig. 2A can be reasonably well described by n^4 kinetics in much the same way as in frog fibres⁷. Finally, the outward current showed a reversal potential which became more positive when the external potassium concentration was increased. It seems, therefore, that the biochemical inhomogeneity previously described for sodium channels in mammalian nodal membrane by Ritchie and Rogart⁸ is accompanied by a corresponding complementary inhomogeneity in the distribution of potassium channels. Thus, virtually all the sodium channels are located in the nodal region whereas most of the potassium channels are located in the internodal region. The significance of this uneven distribution of potassium channels remains unclear.

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Tumour promoter uncouples β -adrenergic receptor from adenyl cyclase in mouse epidermis

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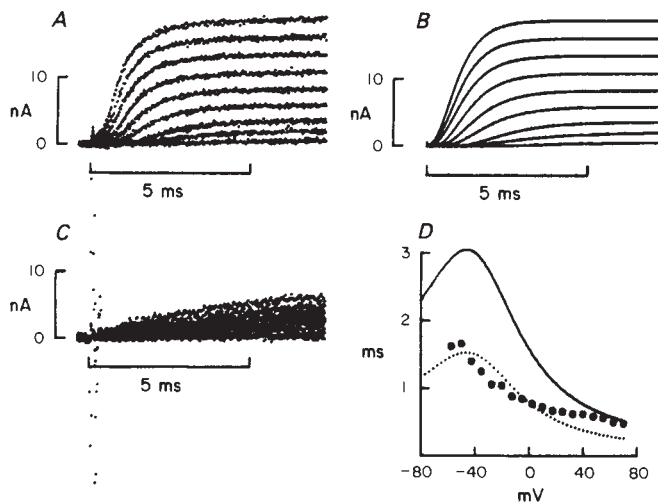


Fig. 2 Kinetics of the late outward current. A shows the effect of applying 100 nM tetrodotoxin on the family of currents in Fig. 1B: the early sodium currents were blocked and only the late outward currents were left. The currents in A were reconstructed in B using the expression $I = I_0[1 - \exp\{-(t - t_D)/\tau_n\}]^4$ with $I = 0$ for $t < t_D$. The voltage dependence of τ_n in the same experiment is shown in D (●). Also shown is the theoretical time constant (solid line) for the frog potassium current at 22 °C using the rate constants from ref. 6; for comparison with the rabbit data this curve has been scaled by a factor of 0.5 (broken line). C shows that the outward current is sensitive to internal Cs and TEA. The records were taken 10 min after changing the end pool solution in A from KCl to 80 mM CsCl and 80 mM TEA-Cl. Temperature, 24 °C.

Alterations in β -adrenergic receptor number and function and in the hormonal responsiveness of adenylate cyclase have been observed in transformed cells^{1–3}, and tumours^{4,5}. Phorbol myristate acetate (PMA), a potent tumour promoter in mouse skin, induces a dramatic loss of epidermal responsiveness to catecholamines *in vivo*^{6–8}, although basal levels of cyclic AMP are not affected^{7–9}. In other work we have shown that PMA treatment does not alter the number or affinity of epidermal β -receptors, although accumulation of cyclic AMP in response to isoprenaline injection is sharply inhibited¹⁰. Evidence is presented here that PMA exerts this effect by uncoupling epidermal β -receptors from adenylate cyclase.

Maguire *et al.*¹¹, and Lefkowitz and Williams¹² have established several criteria to distinguish well coupled β -adrenergic receptor–adenylate cyclase systems from uncoupled or poorly coupled ones. Well coupled adrenergic systems from many cell types and tissues exhibit higher ratios of K_D (dissociation constant for agonist binding) to K_{act} (activation constant or concentration of agonist required to give 50% maximal response) than do poorly coupled systems. Agonist binding is decreased in coupled systems (K_D is increased) by the presence of guanine nucleotides, whereas guanine nucleotides do not affect agonist binding in uncoupled systems. Coupled systems often show lower K_D values and lower Hill coefficients for agonists than do uncoupled systems¹³.

We have measured cyclic AMP accumulation and β -receptor binding by the β -agonist (–)isoprenaline in epidermal homogenates from untreated and PMA-treated mice. Mouse epidermal β -adrenergic receptors have been previously identified and characterised in our laboratory¹⁰, using the labelled

β -antagonist ^3H -labelled (-)-dihydroalprenolol (DHA)¹⁴. These receptors exhibit rapid and reversible equilibrium binding, saturability and stereospecificity for the L configuration of antagonists¹⁰.

Female 8-week-old Ha/ICR mice (Sprague-Dawley) were shaved, treated with 10 μg PMA (obtained from P. Borchert, Chemical Carcinogenesis, Eden Prairie, Minnesota) in 0.2 ml acetone or with 0.2 ml acetone alone and killed 12 h later. This interval was chosen because Grimm and Marks have shown the loss of epidermal β -adrenergic responsiveness to be maximal 12 h after PMA treatment⁶. Back skins were removed, stretched on cards epidermis side up, and frozen in liquid N_2 . Epidermis was removed by scraping as previously described⁸. Epidermal homogenates were prepared by homogenisation in a loose Dounce homogeniser (15 strokes by hand) in cold phosphate-buffered saline (PBS), pH 7.2. Agonist binding to homogenates was determined by competition with ^3H -labelled (-)-dihydroalprenolol (NEN) as described in the legend to Fig. 3, and cyclic AMP was measured by radioimmunoassay⁸.

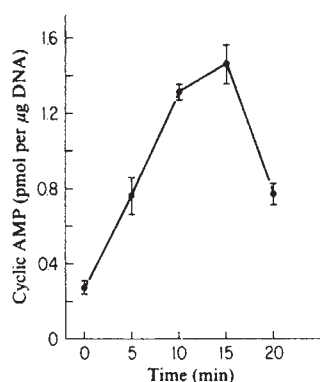


Fig. 1 Cyclic AMP accumulation in mouse epidermal homogenates treated with isoprenaline *in vitro*. Pooled epidermal homogenates were prepared from 15 skins as described in the text using 3 ml PBS per epidermis. In 25 ml flasks 2.4 ml of homogenate and 0.3 ml GTP (3 mM) were incubated at 37 °C for 15 min with shaking. Isoprenaline (30 μl) was added for a final concentration of 5×10^{-3} M and the flasks were incubated with shaking at 37 °C for the indicated times, at which point 0.2 ml 100% trichloroacetic acid was added. The flask contents were homogenised in a Polytron, the supernatant was extracted with ether and cyclic AMP was determined by radioimmunoassay as previously described⁸. DNA was determined by fluorometric assay²³. Values shown are means $\pm$ s.e.m. of triplicate points.

Incubation of epidermal homogenates *in vitro* with isoprenaline results in an increase in cyclic AMP levels as shown in Fig. 1. The peak at 10–15 min, followed by a decline is characteristic of the *in vivo* epidermal response to intraperitoneal injection of isoprenaline^{6,7}. Figure 2 illustrates the dose-dependent effect of isoprenaline on homogenates from untreated and PMA-treated mice. The maximal stimulation of cyclic AMP accumulation in PMA-treated epidermis is significantly reduced from controls, and requires 10 times the concentration of isoprenaline. The K_{act} is increased approximately fourfold in the treated group.

Effects of GTP on isoprenaline binding to epidermal homogenates are shown in Fig. 3. The binding and activation data for control and PMA-treated epidermis, are summarised in Table 1.

From these data it seems that *in vivo* PMA treatment of mouse skin eliminates the effect of GTP on K_D and the pseudo-Hill coefficient (k_h) seen in control homogenates. In addition, both binding parameters are increased over controls without GTP. Of primary importance is the large drop ($\sim$ sevenfold) in the K_D/K_{act} ratio after PMA treatment.

The concentration of isoprenaline required for 50% displacement of the labelled antagonist is considerably ($\sim 10^2$)

Table 1 Isoprenaline binding and activation parameters for untreated and PMA-treated mouse epidermis

	GTP* (300 μM)	Untreated	PMA-treated†
K_D (mM)	—	0.35	0.72
K_D (mM)	+	1.3	0.79
k_h ‡	—	-0.63	-0.85
k_h	+	-0.80	-0.86
K_{act} (mM)§	+	0.39	1.33
K_D/K_{act}	+	4.33	0.60

* GTP present (+) or absent (—) in the incubation mixture.

† PMA (10 μg in 0.2 ml acetone) applied percutaneously 12 h before death.

‡ Pseudo-Hill coefficient (slopes of plots in Fig. 3c, d).

§ K_{act} was determined only in the presence of GTP.

higher than that seen in other β -receptor systems. Not enough is known about the epidermal β -adrenergic system to properly evaluate this discrepancy, although in other work¹⁰ we found that the apparent K_D for antagonist (dihydroalprenolol) binding (52 nM) was also higher than other reported values^{11,12}. Comparably high levels of isoprenaline were required for stimulation of cyclic AMP accumulation as well as for binding. This may be a property of the *in vitro* epidermal preparation and incubation conditions used.

The agonist binding and activation results presented here provide evidence for a β -receptor-adenylate cyclase 'uncoupling' action by PMA. These results are consistent with data obtained using the uncoupling agent filipin^{15,16} and an uncoupled clone of S49 mouse lymphoma¹⁷. Several laboratories have found that incubation of cells with PMA results in a number of cell surface changes^{18–20}. Lee and Weinstein²¹ have shown that PMA interferes with binding of epidermal growth factor to HeLa cell membranes. An uncoupling mechanism for the loss of β -adrenergic responsiveness in mouse epidermis by PMA represents another facet in the action of phorbol ester on plasma membranes.

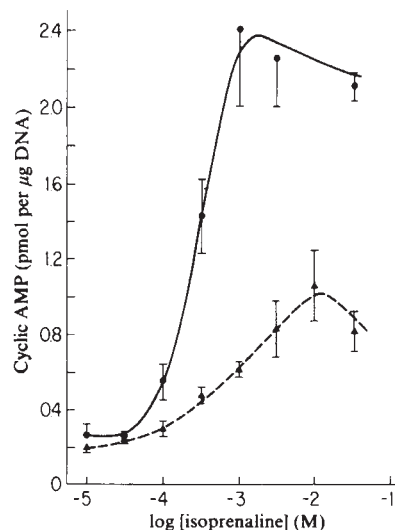


Fig. 2 Effect of PMA on isoprenaline-stimulated cyclic AMP accumulation in mouse epidermis *in vitro*. Epidermal homogenates were prepared from back skins of untreated (●) or PMA-treated (▲) mice killed 12 h after treatment. Incubation and cyclic AMP determination were carried out as described in the legend to Fig. 1, except that the concentration of isoprenaline was varied as indicated and the time of incubation was 12 min. Values shown are means $\pm$ s.e.m. of triplicates.

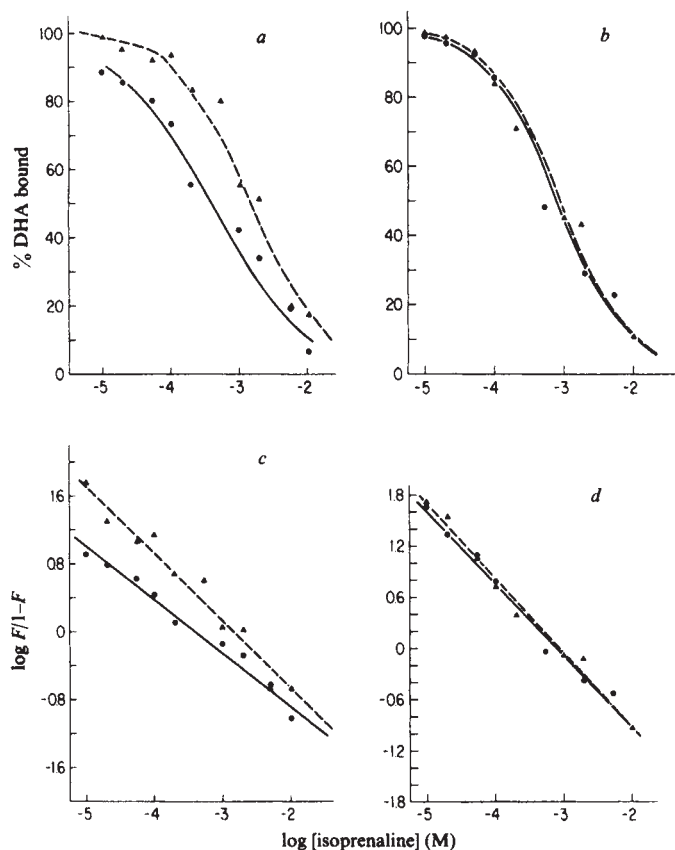


Fig. 3 Effects of GTP on binding of (–)isoprenaline to mouse epidermal β -receptors. Isoprenaline binding was measured by competitive displacement of ^3H -labelled DHA in the presence ($\blacktriangle$) and absence ($\bullet$) of 300 μM GTP in untreated (a) and in PMA-treated (b) epidermis. Hill plots²⁴ for agonist binding in the presence ($\blacktriangle$) and absence ($\bullet$) of GTP for untreated (c) and PMA-treated (d) epidermis were calculated from the binding data. In triplicate tubes 0.2 ml of epidermal homogenate were incubated at 37 °C in a total volume of 0.5 ml containing PBS, ^3H -labelled DHA (5 pmol, 0.25 μCi) and (–)isoprenaline at indicated molar concentrations. GTP (0.15 μmol) was included in some experiments; (–)propanolol (5 nmol) was added in duplicate tubes for determination of nonspecific binding. After 1 h, binding was terminated by addition of 2 ml ice-cold PBS and tube contents were immediately filtered through GF/D glass fibre filters (Whatman). The filters were thoroughly washed under vacuum with cold PBS, dried, and counted in 10 ml Aquasol (NEN). Counts from tubes containing (–)propanolol were subtracted from total counts to give specific binding. Non-specific binding of DHA represented between 30–50% of total counts. The concentration of DHA used (10 nM) gives 10% saturation of binding sites¹⁰.

Recent work on the β -adrenergic receptor–adenylate cyclase system suggests that the transduction of hormone binding into cyclase activation involves a ‘migration’ of receptors in a fluid membrane²². Phorbol ester, a lipid soluble membrane active compound, may interfere with the transduction or coupling process by altering the fluid or dynamic state of the membrane. The β -adrenergic uncoupling effect demonstrated here may be nonspecific and representative of a general loss or distortion of membrane function (in this case response to hormones) caused by PMA. The significance of the uncoupling effect for tumour promotion *in vivo* is indicated by a correlation between loss of adrenergic response and tumour promoting potency of a number of compounds⁶. An investigation of the coupling efficiency of epidermis undergoing two-stage chemical carcinogenesis and in skin papillomas and tumours is under way.

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Host genotype influences immunomodulation by interferon

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Interferon influences both afferent and efferent pathways of delayed-type hypersensitivity (DH) in the mouse. In animals previously sensitised to picryl chloride, sheep red blood cells (SRBC) or Newcastle disease virus (NDV), and treated with interferon just before challenge with any of these antigens, the antigen-elicited reaction, as measured by the ear-swelling (picryl chloride) or footpad swelling (SRBC and NDV) test, is either decreased or completely inhibited, depending on the dose of interferon administered^{1,2}. In addition to this action on expression of the sensitised state, interferon decreases or inhibits sensitisation to SRBC or NDV when administered 24 h before immunisation^{2,3}. These effects were recently confirmed using electrophoretically pure mouse interferon, thus ruling out the possibility that they are caused by other proteins present in the previously used partially purified interferon preparations⁴. For the effect on sensitisation, the timing of interferon administration is crucial, and when interferon is administered a few hours after the antigen, sensitisation can actually be enhanced, as reported here; the enhancement of sensitisation by interferon is influenced by the dose of antigen and by the genotype of the mice that are sensitised.

Sensitisation with sheep erythrocytes was achieved by the method of Lagrange *et al.*⁵. Briefly, mice are sensitised by an intravenous (i.v.) injection of 10^6 SRBC, followed 4 days later by challenge with 10^8 SRBC in 40 μl of phosphate-buffered saline into the left hind footpad. Footpad swelling is measured 24, 48 and 72 h later with calipers and expressed as the difference in thickness between right (unchallenged) and left foot. Usually, 48-h values are 40–80% higher than 24-h values; at 72 h footpad swelling has significantly decreased.

The interferon used for these experiments was prepared in monolayer cultures of C-243 cells, induced with NDV after priming^{6,7}. It was then purified and concentrated either on blue Sepharose⁸ or on poly (U) Sepharose⁹, giving preparations with a specific activity of $10^{7.5}$ to 10^8 units per mg. For one experiment (Table 1) electrophoretically pure interferon, of specific

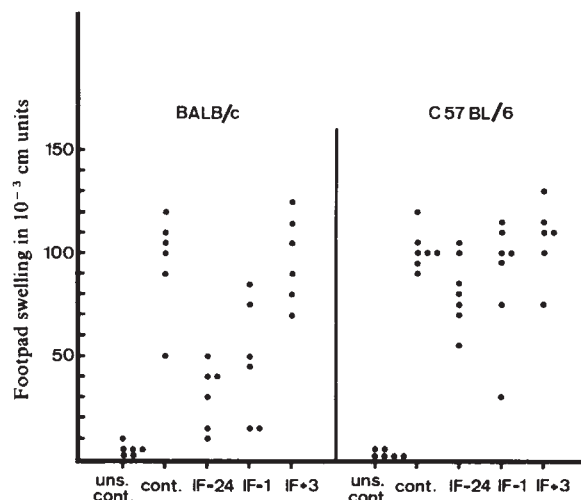


Fig. 1 Effect of timing of interferon administration on sensitisation. Three-month-old female BALB/c or C57BL/6 mice were sensitised with 10^6 SRBC. One group of each strain received an intraperitoneal (i.p.) inoculation of 3.3×10^5 interferon units in 1 ml either 24 h before (–24 h), 1 h before (–1 h) or 3 h after (+3 h) sensitisation. Footpads were challenged 4 days later; each point represents the 48-h reaction of one animal. See Table 2 for analysis of data.

activity 2×10^9 unit per mg, was used¹⁰. All interferon units are expressed in NIH reference units.

When interferon is given 24 h before immunisation with SRBC, inhibition of sensitisation is more pronounced in BALB/c mice than in C57BL/6 mice, and when it is given only 1 h before SRBC, inhibition of sensitisation no longer occurs in C57BL/6 mice whereas it is still significant in BALB/c mice. These results are summarised in Fig. 1 and Table 2. When interferon is administered 3 h after sensitisation, no significant difference in reaction after footpad challenge is observed between interferon-treated and controls either in BALB/c or C57BL/6 mice, provided they have been sensitised with an optimal dose of antigen (10^6 SRBC) (see also Fig. 1). However, if a sub-optimal dose of antigen is used (10^5 SRBC), a stimulating effect of the subsequent interferon treatment is observed, and this enhancement is more pronounced in C57BL/6 than BALB/c mice (Fig. 2a, Table 2). The difference in reaction to interferon between mice of the two strains is also evident with a dose of antigen that normally does not result in sensitisation. BALB/c mice sensitised with $10^{4.3}$ SRBC and challenged 4 days later do not develop significant footpad swelling, regardless of whether they have been treated with 3.3×10^5 units of interferon 3 h after immunisation. C57BL/6 mice, on the other hand, develop significant footpad swelling if they have been treated with interferon 3 h after immunisation with $10^{4.3}$ SRBC (Fig. 2b, Table 2). Thus, the immunosuppressive effect of exogenous interferon on the afferent pathway of DH to SRBC is more

pronounced in BALB/c than C57BL/6 mice, whereas the immunostimulating effect is more pronounced in C57BL/6 than BALB/c mice.

A striking difference between mice of the two genotypes also exists when the influence of interferon on expression of DH to SRBC is examined. When given a few hours before footpad challenge, interferon inhibits the expression of DH in both strains. However, it is less active in C57BL/6 mice. For example, a dose leading to a pronounced inhibition of footpad swelling in sensitised BALB/c mice remains without significant effect in C57BL/6 mice (Fig. 3, Table 2). About 25 times the amount of interferon used in BALB/c is needed to obtain comparable inhibition of footpad swelling in C57BL/6 (Table 1).

These results have several implications for the immunomodulatory effects of type I interferon. The timing of interferon administration with regard to the time of immunisation is crucial in determining whether inhibition or enhancement of sensitisation will occur. A similar observation was made in a study of the effects of interferon on antibody formation *in vitro*; when interferon is added before the antigen, inhibition of antibody formation is observed, whereas when interferon is added after the antigen, enhancement is sometimes obtained¹¹. In addition to timing, antigen dosage is important, and significant immunoenhancement only results when sub-optimal or normally non-sensitising doses of SRBC are used. In addition to stressing the importance of timing and antigen dose, our results show that immunomodulation by interferon is under the influence of host genotype, as the immunosuppressive effect of interferon on both afferent and efferent pathways of DH to SRBC is less pronounced in C57BL/6 than BALB/c mice, whereas, in contrast, the immunoenhancing effect is more readily obtained in C57BL/6 mice. These observations indicate

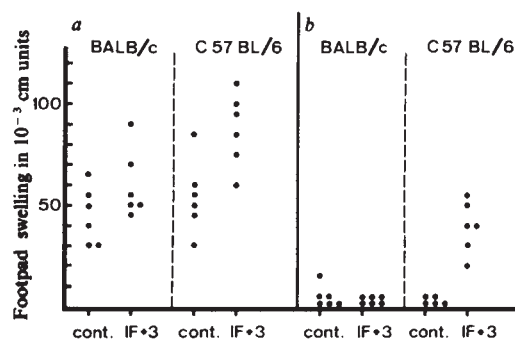


Fig. 2 Influence of antigen dosage and mouse strain on enhancement of sensitisation by interferon. Three-month-old female BALB/c or C57BL/6 mice were sensitised by an intravenous inoculation of either 10^5 (a) or $10^{4.3}$ (b) SRBC. The interferon-treated group received 3.3×10^5 units in 1 ml i.p. 3 h after sensitisation. The footpads were challenged 4 days later; each point represents the reaction of one animal, measured 48 h after challenge. See Table 2 for analysis of data.

Table 1 Effect of interferon on expression of DH to SRBC

BALB/c				C57BL/6			
	No. of mice	24-h footpad swelling in 10^{-3} cm units (mean $\pm$ s.d.)	% Of untreated control		No. of mice	24-h footpad swelling in 10^{-3} cm units (mean $\pm$ s.d.)	% Of untreated control
Controls	6	52.5 $\pm$ 16.0	—		6	65.0 $\pm$ 13.7	—
Interferon treated							
a	6	10.0 $\pm$ 7.0	13 $P < 0.005$		6	40.0 $\pm$ 23.1	63 $P < 0.025$
b	6	28.3 $\pm$ 10.3	54 $P < 0.01$		6	54.0 $\pm$ 29.4	83 NS
c	6	32.5 $\pm$ 16.3	62 $P < 0.05$		6	56.0 $\pm$ 6.0	87 NS

The experimental protocol was essentially identical to that followed for the experiments summarised in Fig. 3, except that electrophoretically pure interferon (IF) was used. IF a: 2.2×10^5 units per ml; b: 4.4×10^4 units per ml; c: 8.8×10^3 units per ml. P , Level of significance of difference with control. NS, Not significant. Each animal received 1 ml of interferon.

Table 2 Statistical analysis of the data represented in Figs 1-3

Fig. 1		BALB/c			C57BL/6		
	Mean $\pm$ s.d.	% Of control			Mean $\pm$ s.d.	% Of control	
Controls	95.8 $\pm$ 24.5	—	—		101.4 $\pm$ 9.4	—	—
IF-24 h	30.8 $\pm$ 15.6	32	$P < 0.005$		81.4 $\pm$ 17.2	80	$P < 0.025$
IF-1 h	47.5 $\pm$ 29.2	50	$P < 0.01$		89.2 $\pm$ 29.0	88	NS
IF+3 h	97.5 $\pm$ 21.1	102	NS		106.6 $\pm$ 18.3	105	NS

Fig. 2		BALB/c		Mean $\pm$ s.d.	C57BL/6		
Sensitised with 10^5 SRBC			Control				
			Interferon	40.0 $\pm$ 23.0	—		
				60.0 $\pm$ 17.0	NS		
		C57BL/6	Control	54.1 $\pm$ 18.2	—		
			Interferon	87.5 $\pm$ 18.1	$P < 0.005$		

Sensitised with $10^{4.3}$ SRBC		C57BL/6		Mean $\pm$ s.d.	C57BL/6		
			Control	2.0 $\pm$ 2.7	—		
			Interferon	35.8 $\pm$ 19.6	$P < 0.005$		

Fig. 3		BALB/c			C57BL/6		
		% Of control			% Of control		
24 h	Cont.	45.6 $\pm$ 20.7	—	—	59.6 $\pm$ 26.4	—	—
	IFa	11.1 $\pm$ 8.7	24	$P < 0.005$	40.4 $\pm$ 24.9	68	$P < 0.01$
	IFb	26.7 $\pm$ 24.5	59	$P < 0.01$	47.7 $\pm$ 25.8	80	NS
48 h	Cont.	82.5 $\pm$ 24.0	—	—	93.2 $\pm$ 33.3	—	—
	IFa	45.2 $\pm$ 20.5	55	$P < 0.005$	82.8 $\pm$ 33.0	89	NS
	IFb	54.1 $\pm$ 31.6	66	$P < 0.005$	89.5 $\pm$ 26.0	96	NS

P, Level of significance of difference with control. NS, Not significant.

that, in addition to genes influencing the production of interferon^{12,13}, there are genes in the mouse that influence the action of interferon on cells of the immune system; recombinant inbred and congenic lines derived from BALB/c and C57BL/6 mice will be instrumental for further genetic analysis of this phenomenon.

There are other examples of genes influencing interferon action. In the mouse, the *Mx* locus was recently shown to influence interferon action specifically and exclusively on orthomyxovirus replication (O. Haller, personal communication). In man, a gene (or genes) on chromosome 21 influences antiviral activity, and cells trisomic for this chromosome are more sensitive to the antiviral activity of interferon and also to its cell multiplication inhibitory activity¹⁴⁻¹⁶. Recently, a correspond-

ing gene has been localised in the mouse on chromosome 16 (L. Epstein, personal communication, and D. Slate, personal communication). This gene is probably not involved in the effects of genotype interferon action described here because when we compared mouse embryo fibroblast cultures from BALB/c and C57BL/6 origin for their sensitivity to the antiviral effect of interferon as measured by inhibition of VSV replication, no difference was found between the two genotypes (unpublished observations). Different sensitivities to the action of interferon therefore do not seem to be a general characteristic of all BALB/c and C57BL/6 cells. It is more likely that the difference is expressed at the more specialised level of T-cell, B-cell or macrophage function, and *in vitro* experiments comparing cells of both genotypes may clarify this point.

In addition to their theoretical interest, our results have a practical implication. Similar differences in immunomodulation by interferon according to genotype, if found in man, could explain the significant individual variations in the clinical effects of interferon that have been observed.

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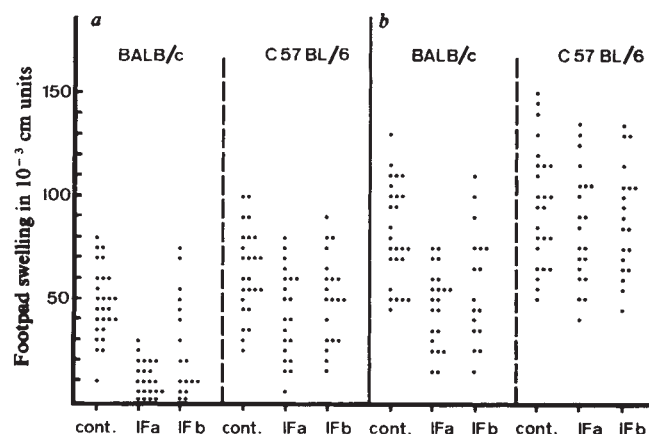


Fig. 3 Immunosuppressive effect of interferon on expression of DH to SRBC in BALB/c and C57BL/6 mice. The results of three different experiments are combined. Three-month-old female BALB/c and C57BL/6 mice were sensitised by an i.v. inoculation of 10^6 SRBC. Four days later and 3 h before footpad challenge, the interferon-treated group received an i.p. inoculation of 1 ml containing 3.3×10^5 interferon units (IFa), 3.3×10^4 interferon units (IFb) or phosphate-buffered saline (cont.). Footpad swelling was measured 24 h (a) and 48 h (b) after challenge. Each point represents the footpad swelling of one mouse. The means $\pm$ s.d. of the plotted values are given in Table 2.

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Herpes simplex virus depresses antibody production by affecting T-cell function

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Many viruses enter lympho-reticular cells during pathogenesis and thereby induce immunosuppression¹, which is of practical importance in that it may be related to overall virulence². Immunosuppression may result from a selective infection, as viruses often show an affinity for different lymphocyte subpopulations: Epstein-Barr virus, for example, infects only a small percentage of B cells³. We reported previously that herpes simplex virus (HSV) type 1 suppressed the induction of an antibody response to diphtheria toxoid in cultures of human tonsil cells, and that this seemed to result from the infection of a small percentage of T lymphocytes⁴. However, as fully infectious virus was used in these experiments, it had probably spread from cell to cell in the course of the culture, so complicating the interpretation of the results. Accordingly, we have now re-investigated the mechanism of immunosuppression using temperature-sensitive (*ts*) mutants which fail to complete their growth cycle in the conditions selected for antibody synthesis. In this study, mutants *ts*B, *ts*D and *ts*F, derived from HSV type 1 strain 17, and *ts* 9, derived from HSV type 2 HG 52, were used. The results suggest that the immunosuppression is due to the selective infection by the viruses of helper T cells.

In four experiments a representative example of which is presented in Table 1, the HSV type 1 mutants *ts* B, *ts* D and *ts* F consistently suppressed the synthesis of both specific antibody and immunoglobulin as effectively as did wild-type HSV 1 and HSV 2. In contrast, the HSV type 2 mutant *ts* 9 had little effect. In these experiments, virus was added 24 h after antigen stimulation. Virus assays carried out in all experiments showed that the temperature-sensitive mutants grow in lymphocytes cultured at 31°C but not at the restrictive temperature of 37°C.

Susceptibility to the immunosuppressive effects of the virus was of limited duration, for *ts* F failed to affect antibody synthesis at 37°C when added 72 h or more after induction of the response. This is in keeping with our original observations⁴. After 7 d, the viability of cells in cultures infected with *ts* F, as measured by trypan blue exclusion, was 65% compared with 73% in uninfected cultures, indicating that immunosuppression did not result from an excessive death of lymphocytes.

Table 1 Immunosuppression by *ts* mutants of herpes simplex virus

Virus added	Diphtheria toxoid antibody (U ml ⁻¹)	IgM (μg ml ⁻¹)	IgG (μg ml ⁻¹)
Nil	2.13 ± 0.11	29.5 ± 0.7	57.0 ± 8.4
Type 1 <i>ts</i> ⁺	0.34 ± 0.02	9.8 ± 1.3	25.6 ± 2.1
<i>ts</i> B	0.36 ± 0.02	2.15 ± 0.8	26.5 ± 7.7
<i>ts</i> D	0.45 ± 0.03	9.5 ± 0.7	35.5 ± 3.5
<i>ts</i> F	0.39 ± 0.06	6.8 ± 2.1	37.0 ± 4.9
Type 2 <i>ts</i> ⁺	0.35 ± 0.03	1.9 ± 2.6	36.0 ± 5.6
<i>ts</i> 9	1.45 ± 0.37	24.4 ± 3.1	60.25 ± 3.1

Human tonsil lymphocytes were stimulated with diphtheria toxoid antigen and infected 24 h later with wild-type HSV or *ts* mutants at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU) per cell. The cultures were maintained for 7 days at 37°C. The supernatants were assayed for specific antibody to diphtheria toxoid by double antibody radioimmunoassay and for Ig classes by solid phase radioimmunoassay⁴. Values are mean ± s.e.m. of duplicate assays on triplicate cultures from a single tonsil.

The temperature-sensitive block in the mutant *ts* F growth cycle occurs late, allowing the synthesis of structural proteins but not infectious progeny; infected cells could therefore be counted by indirect immunofluorescence in the absence of virus spread. As shown in Table 2, using *ts* F as inoculum, the proportion of infected cells in the population did not exceed 3.2%. Infectious centre assays carried out at 31°C on cultures infected at 37°C with *ts* D or *ts* F which induce immunosuppression and *ts* 9 which does not, showed that less than 1% of the cells supported virus growth. In further experiments, subpopulations of lymphocytes were infected selectively with *ts* F to determine whether the effect on B-lymphocyte activity was direct or indirect. This necessitated a departure from the standardised method described for immunoglobulin synthesis to a micro-culture method using pokeweed mitogen (PWM)⁵, because of the small numbers of cells available after separation. These experiments showed that only in cultures in which T lymphocytes had been infected was immunoglobulin synthesis impaired. In cultures in which uninfected T lymphocytes were cultivated with infected B lymphocytes, immunoglobulin synthesis was not significantly depressed (Table 3). Furthermore, the addition of uninfected T lymphocytes to cultures containing infected T and B lymphocytes, 2 h after mitogen stimulation, partially restored immunoglobulin synthesis (Table 4). Complete restoration was not achieved because the optimal cell concentration for synthesis was disturbed by these manipulations⁶.

Table 2 Per cent tonsil cells infected by *ts* mutants of HSV

Days post-infection		0	1	2	3	7
Mutant	Assay					
<i>ts</i> D	ICA	0.01	0.11	0.014	0.12	0.0
<i>ts</i> F	ICA	0.01	0.10	0.016	0.05	0.0
<i>ts</i> 9	ICA	0.01	0.02	0.013	0.01	0.0
<i>ts</i> F	IF	1.1	3.2	2.1	1.8	1.0

Infectious centre assays (ICA) were carried out on monolayers of baby hamster kidney (BHK) cells. Tonsil cells were infected initially with virus for 2 h at 37°C and cultured in the conditions described in Table 1 legend. Before assay the cells were washed three times in L15 medium, and incubated for 1 h at 37°C in medium containing anti-HSV antibody to neutralise extracellular virus, before washing a further three times in L15 at 4°C. The cells were resuspended at concentrations of 10–10⁶ cells ml⁻¹ in L15 medium containing 0.6% carboxymethylcellulose; 1-ml aliquots were added to BHK monolayers grown in 12-mm diameter, 24-welled plastic plates (Linbro, Flow Laboratories) which were then centrifuged at 300g for 10 min at 4°C. After 3 d incubation at 31°C the plates were fixed with 10% formal saline, stained with crystal violet and the number of plaques counted. Figures are mean of duplicate observations for the percentage of lymphocytes forming plaques. Immunofluorescence (IF) was carried out on cell samples collected daily from bulk cultures. The cells were washed twice in Dulbecco's phosphate-buffered saline, pH 7.4, transferred to Teflon-coated glass slides (Flow Laboratories), fixed in acetone for 15 min at -20°C and stored at -20°C. The preparations were stained with rabbit antisera to HSV type 1 which had been grown in rabbit kidney (RK13) cells, followed by fluorescein-conjugated goat anti-rabbit serum (Behringwerke) and counterstained with rhodamine-conjugated bovine serum albumin (Microbiological Associates). Preparations were mounted in buffered glycerol and examined under a Zeiss microscope (universal) fitted with a Ploem illumination system with separate excitation for the two fluorochromes. Cells were counted in non-overlapping fields until the total number in duplicate samples exceeded 1,000.

As expected, B lymphocytes cultured in isolation failed to synthesise immunoglobulin (Tables 3, 4), indicating that immunoglobulin synthesis in response to PWM is T-cell dependent⁶. The addition of 'T-helper factor' restored immunoglobulin synthesis by isolated infected or uninfected B lymphocytes to an equivalent extent (Table 4). These results confirm that HSV-induced immunosuppression results from a loss of T-helper function.

Table 3 Effect of *ts* mutants of herpes simplex virus on function of lymphocyte subpopulations

Combinations of lymphocyte subpopulations cultured	IgG ($\mu\text{g ml}^{-1}$)
Unseparated	61.75 $\pm$ 8.5
Unseparated (<i>ts</i> F)	<3.0
T+B	47.75 $\pm$ 4.1
T (<i>ts</i> F)+B	13.88 $\pm$ 5.0
T+B (<i>ts</i> F)	32.5 $\pm$ 8.48
T (<i>ts</i> F)+B (<i>ts</i> F)	<3.0
B alone	<3.0

Lymphocyte subpopulations were separated by the rosetting method of Gmelig-Meyling and Ballieux⁷. B-lymphocyte preparations obtained contained 98% immunoglobulin-bearing cells⁸, whereas T lymphocytes had <1%. Cultured cells were stimulated with 0.1 ml pokeweed mitogen per 1×10^7 cells at 37°C for 16 h at a concentration of 1×10^6 cells ml^{-1} in Falcon flasks, in a growth medium of RPMI 1640 (bicarbonate buffered), 10% fetal calf serum and 100 U ml^{-1} each of penicillin, streptomycin and mycostatin. The cells were then washed and infected by exposure to *ts* F mutant of HSV at an MOI of 1.0 PFU per cell for 2 h at 37°C. Extraneous virus was removed by washing three times. Cells restimulated by the addition of 2 μl per well of pokeweed mitogen, prepared by standard methods, were cultured in growth medium in wells of microtitre plates at a concentration of 2×10^5 cells per well. The ratio of T lymphocytes to B lymphocytes in recombination experiments were the same as the ratio in the unseparated cultures, namely 1:2. The cells were cultured at 37°C in an atmosphere of 5% CO₂ in air for 7 days. Supernatants were collected and assayed for immunoglobulin by solid phase radioimmunoassay.

Table 4 T cells or T-cell factor restores immunoglobulin synthesis by B lymphocytes exposed to HSV

Combination of lymphocyte subpopulations cultures	IgG ($\mu\text{g ml}^{-1}$)
Expt 1	
T+B	40.0 $\pm$ 8.3
T(<i>ts</i> F)+B(<i>ts</i> F)	<3.0
T(<i>ts</i> F)+B(<i>ts</i> F)+T	16.25 $\pm$ 2.9
T(<i>ts</i> F)+B(<i>ts</i> F)+T(<i>ts</i> F)	5.0 $\pm$ 1.0
Expt 2	
B alone	0.33 $\pm$ 0.2
B lymphocytes + T-helper factor	7.4 $\pm$ 1.4
B lymphocytes + T-helper factor + HSV	6.6 $\pm$ 1.4
T-helper factor alone	<0.01

Expt 1: culture conditions were as described in Table 3 legend. In these cultures, 5×10^4 infected T lymphocytes were incubated with 1×10^5 infected B lymphocytes for 2 h after adding PWM. 5×10^4 of either uninfected or infected T lymphocytes were added. Supernatants were collected after 7 days and assayed for immunoglobulin by solid phase radioimmunoassay. **Expt 2:** 2×10^5 cells were cultured with PWM in microtitre plates for 7 days. B lymphocytes were cultured in a mixture of one part of a 24-h culture supernatant from PWM-stimulated, unseparated cells from the same tonsil and two parts fresh medium. HSV at an MOI of 0.1 was added to the infected cells on day 1 of culture.

Table 5 Failure of ultracentrifuged supernatants from HSV-immunosuppressed tonsil cultures to depress immunoglobulin synthesis in fresh cultures

Culture	IgG ($\mu\text{g ml}^{-1}$)
Immunised	15.5 $\pm$ 4.0
Immunised + immunosuppressed supernatant	18.2 $\pm$ 5.2
Immunised + control supernatant	22.0 $\pm$ 6.5

Supernatants from 7-day-old tonsil cultures immunosuppressed by HSV or from non-immunosuppressed controls were centrifuged at 25,000g for 1 h. New tonsil cells were cultured in the conditions described in Table 1 legend in a mixture of one part of supernatant and two parts of fresh medium. All cultures were assayed after 7 days for immunoglobulin production and virus infectivity. In addition, the original supernatants were tested for infectious virus particles. All virus assays were negative.

We have not detected significant amounts of interferon in cultures of tonsil cells infected with HSV⁴; furthermore, the medium in which infected cells have been cultured and in which free virus has been removed supports normal levels of antibody synthesis by fresh tonsil cells (Table 5).

Our findings indicate that HSV can suppress the antibody response of B lymphocytes to diphtheria toxoid by infecting only a small population of T cells and that this suppression does not depend on a complete cycle of virus replication. Beyond this the mechanism is unclear, for HSV type 1 mutants blocked early (*ts* B, *ts* D) or late (*ts* F) in infection at 37°C were equally immunosuppressive. In contrast, the early-block mutant of HSV type 2, *ts* 9, did not immunosuppress. Because *ts* 9 causes less disruption of host cell metabolism than the other mutants (H. Marsden, personal communication), it suggests that a very early event, such as depression of host cell protein synthesis, may be concerned in the mechanism of HSV-induced immunosuppression.

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Lymphocyte differentiation and major histocompatibility complex antigen expression in the embryonic thymus

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During embryogenesis, stem cells migrate from the bloodstream into the thymic rudiment where they proliferate and differentiate into T lymphocytes. The epithelial cells of the thymic stroma may influence these processes by providing hormonal and/or contact stimuli to the developing lymphoblasts^{1,2}. Recently, it has been shown that T cells 'learn' to recognise the major histocompatibility complex (MHC) antigens during thymic lymphopoiesis and become MHC-restricted. Their subsequent response to other antigens can only occur in the context of MHC antigens of the haplotype encountered in the thymus³. Little is known, however, of antigen expression on the thymic stroma which may provide the reference framework on which this MHC restriction is based. In this study we use monoclonal antibodies to show that antigens of the K and I regions of the MHC are detectable on cells of the embryonic mouse thymic stroma from around the 14th day of gestation, just when lymphocyte differentiation is commencing. Furthermore, I-region antigen (Ia antigen) expression is probably limited to thymic epithelium at this stage of gestation and we have not detected Ia on other epithelial tissues of the pharyngeal complex. This pattern of expression is consistent with a role for the thymic stroma in MHC restriction, perhaps by the selection of lymphoid cells for survival on the basis of their recognition of stromal MHC determinants.

Primary cultures of thymus and other tissues removed from CBA/ca (H-2^k), C57BL/10(H-2^b) and BALB/c (H-2^d) embryos were set up in multiwell plates (for details see legend to Table 1). Within 24 h of explantation, cultures were examined for expression of MHC and Thy-1 antigens by an indirect immunofluorescence assay using monoclonal antibodies followed by purified, class-specific, Fab preparations of goat antibodies to mouse IgG₂ or goat antibodies to mouse IgM conjugated with fluorescein or rhodamine (for details see legend to Fig. 1). In this way, the possibility of reactivity to antigens other than those under investigation is minimised and specificity is obtained as evidenced by the complete absence of labelling on control cultures exposed to conjugate alone or where tissues from inappropriate haplotypes are employed. The use of short-term cultures allows unfixed, viable cells to be labelled while avoiding changes in cell populations or cell phenotype that might occur in long-term cultures.

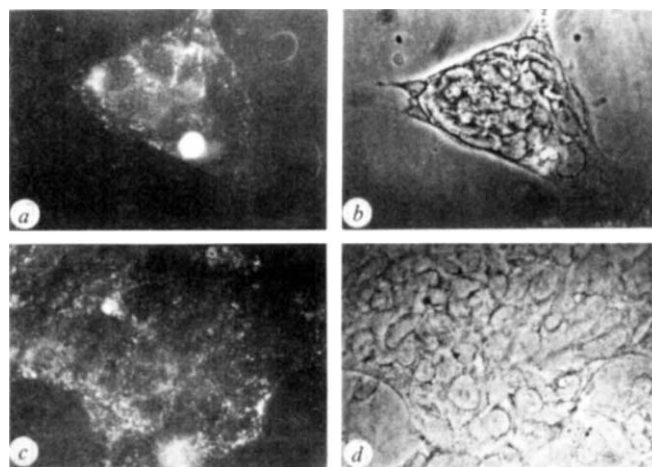


Fig. 1 Immunofluorescence (a) and phase contrast (b) micrographs of an island of epithelial-like cells in embryonic mouse thymus culture treated with monoclonal anti-Ia antibody. Note punctate fluorescent labelling on cell membranes. c and d, Immunofluorescence and phase contrast micrographs of a similar thymus culture labelled with monoclonal anti-H-2K. Antisera, monoclonal (IgG₂) anti-Ia. 17 (I^k) and monoclonal (IgG₂) anti-H-2K^k (both culture supernatants) used at a dilution of 1 in 4 in RF-20 and monoclonal (IgM) anti-Thy-1.2 (F7D5 ascitic fluid) used at a dilution of 1 in 20 were a generous gift from Drs Beverley and Lake, University College, London. Details of the anti-MHC antisera, which originated from the Department of Genetics, Stanford, USA, coded 10-3.6 and 11-4.1 respectively are given in ref. 9. Fluorescein- and rhodamine-conjugated purified class-specific antibodies to IgG₂ and IgM were a generous gift from Drs Cooper and Lawton, Birmingham, USA and have been described in detail elsewhere¹⁰. Before use on embryonic tissues, monoclonal reagents were checked for specificity and reactivity on adult mouse spleen cells (anti-MHC reagents) and on adult mouse and rat thymocytes (anti-Thy-1.2) (data not shown). Labelling of monolayer cultures were carried out by aspiration of medium from culture wells, addition of 20 µl of antiserum dilution and incubation at 37 °C for 30 min. Wells were washed individually by addition and aspiration of 50 µl of RF-20 followed by addition of 10 µl of rhodamine-conjugated, class-specific Fab preparations of goat anti-mouse IgG₂, fluorescein-conjugated goat anti-mouse IgG₂, or rhodamine-conjugated goat anti-mouse IgM (all at 0.5 mg ml⁻¹) as appropriate. Following 30 min incubation at room temperature, individual wells were washed five times with RF-20, fixed with methanol and a small circular cover slip (Chance no. 0, 6 mm diameter) mounted on 30% glycerol in phosphate-buffered saline was dropped into each well. For simultaneous labelling of Ia and Thy-1 antigens, wells were incubated first with a mixture of anti-Ia and anti-Thy-1 antisera followed by rhodamine-conjugated anti-IgM and fluorescein-conjugated anti-IgG₂. All cultures were examined using a Zeiss Universal microscope with epi-illuminator III/RS, Lamp HB0 50 and standard filter sets 9 or 15.

Table 1 MHC and THY-1 antigen expression on embryonic mouse thymus and other organ rudiments

Expt	Age at explantation (d)	Material	Labelling		
			H-2K	H-2I	Thy-1.2
1	13	CBA thymus culture	—	—	NT
		C57BL/10 thymus culture	—	—	NT
2	13	CBA thymus culture	—	—	NT
3	14	CBA thymus culture	NT	+	NT
		BALB/c thymus culture	NT	—	NT
4	14	CBA thymus culture	+	+	NT
		BALB/c thymus culture	—	—	NT
5	14	CBA thymocyte suspension	NT	—	NT
6	14	CBA thymus culture	+	+	—*
		CBA salivary gland culture	+	—	—
7	15	CBA thymus culture	+	+	—*
		CBA head and neck skin culture	+	—	NT
8	15	CBA thymocyte suspension	+	NT	+
9	15	CBA thymus culture	+	+	NT
		CBA lung rudiment culture	+	—	NT
		CBA thyroid culture	+	—	NT

Embryonic thymus and other organ rudiments were dissected from mouse embryos at 13–15 days of gestation (day of plug = day 0), torn open with watchmakers forceps, washed and incubated at 37 °C for 30 min in 0.25% trypsin. Trypsinisation was terminated by centrifugation through a large volume of RPMI 1640 containing 10% fetal calf serum (RF-10) and the products of 10–15 organ rudiments resuspended in 200 µl of the same medium. Aliquots of cell suspension (40 µl) were placed in the wells of Sterilin immunofluorescence trays and cultured overnight. Suspensions of embryonic thymocytes were obtained just before assay by teasing organ rudiments in a small pool of medium. NT, not tested; +, labelled; —, not labelled.

* Double labelling experiments indicated the presence of some Thy-1.2-positive, Ia-negative cells; see text.

The results summarised in Table 1 indicate that K and I-region products of the MHC cannot be detected on cultured stromal elements of day 13 embryonic mouse thymus, but they are readily demonstrable by day 14 of gestation. The majority of labelled cells are located in cell aggregates and in the outgrowths derived from them (Fig. 1). These aggregates represent portions of thymus incompletely dispersed by the disaggregation procedure. We have examined these cell aggregates by transmission electron microscopy and find that they contain many epithelial cells characterised by tonofibrils and intercellular junctions. In

preliminary studies, using ferritin-conjugated anti-mouse Ig antibody to detect monoclonal anti-Ia antibody binding in the electron microscope, we also find that ferritin labelling of epithelial cells can be demonstrated in the appropriate (H-2^k) strain. In view of these results, it seems likely that many, although not necessarily all, of the aggregate cells expressing K and I-region products from the 14th day of gestation onwards are epithelial cells.

Thy-1-positive cells are detectable within and outside cell aggregates, but in double labelling experiments using anti-IgG₂-fluorescein to detect binding of anti-Ia antibody and anti-IgM-rhodamine to detect binding of anti-Thy-1 antibody, Ia- and Thy-1-positive cells are found to be distinct entities. Suspensions of lymphoid cells prepared by 'teasing' 14-day embryo thymuses are Thy-1 positive, but Ia-negative, when treated with the same reagents. It seems likely, therefore, that most, if not all of the Thy-1-positive cells detected within and outside cell

aggregates are lymphoid cells. Although we cannot exclude the possibility that some thymic stromal cells are Thy-1-positive, as suggested in a previous study using alloantisera on adult thymus cultures⁴, our results do indicate that Ia-positive, embryonic thymic epithelial cells are Thy-1-negative. In this context, it may be noted that the stromal cells of our cultures are heterogeneous since, outside aggregates, long spindle-shaped cells are Ia-negative.

In contrast to cultures of thymus, which express antigens of both K and I regions, epithelial tissues derived from salivary gland, head and neck skin, thyroid and lung rudiments, while expressing K region antigen, do not express Ia. This selective expression of Ia, in the site of T-cell differentiation, strengthens the argument that MHC antigens on thymic epithelial cells play a role in the development of restricted antigen recognition by T cells. In particular, since MHC restriction of the various T-cell subsets involves I as well as K and D-region antigens^{3,5,6}, it would be necessary that thymic epithelial cells express Ia antigens as well as the more ubiquitous K and D-region products from the commencement of lymphopoiesis. The present findings provide direct evidence that this is the case. Further analysis, however, is required to clarify the MHC status of non-epithelial stromal cells which could also contribute to this process. In accordance with our findings in the mouse embryo, the use of heterologous antisera on frozen sections has indicated the presence of Ia-like antigens in the adult guinea pig thymus⁷. Similarly, the expression of K and I region-products on thymic epithelial cells of the postnatal mouse has now been reported⁸.

The precise means whereby interactions between lymphocytes and embryonic thymus stromal cells take place is unclear. In view of the absence of Ia on the differentiating T-lymphoid cells and the corresponding absence of Thy-1 on the thymic epithelial cells, it would seem unlikely that interaction is facilitated by the mutual expression of these surface components on lymphoid and epithelial elements respectively. It is also unlikely that Ia expression on thymic epithelial cells is involved in the process of stem cell migration to the thymus, since migration is initiated some days before we can detect Ia expression. A possible role for epithelial Ia antigen in the initiation of stem cell proliferation following their entry into thymic rudiment is, however, conceivable. Nonetheless, the major function of MHC expression on thymic epithelial cells probably relates to the acquisition of MHC restriction by T cells.

Thus our studies show that K and I-region products of the MHC can be detected on cultures of thymic stromal cells from as early as the 14th day of gestation. While the first lymphoid stem cells probably enter the thymic rudiment from about the 11th day of gestation, the major proliferative burst is occurring from about the 14th day onwards. In the context of MHC restriction, it may be significant that antigens of the MHC regions involved in this process become apparent on the thymic epithelium at this time.

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V-J joining of immunoglobulin κ genes only occurs on one homologous chromosome

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In general, heterozygous animal cells express both alleles at a particular locus. The only exceptions are cells of XX genotype after inactivation of one X chromosome¹, and immunoglobulin-producing cells; in each case only one of the two alleles is expressed in differentiated cells and their progeny². This phenomenon, termed allelic exclusion, has been described for several mammalian species including man and mouse. It has been shown that the variable (V) and constant (C) region genes of immunoglobulins undergo a rearrangement during ontogeny^{3,4}. We wished to test whether allelic exclusion in B cells could be the consequence of V- and C-region rearrangement on one of the two homologous chromosomes only. For that reason we chose to analyse the rearrangement of immunoglobulin light chain genes in normal B lymphocytes isolated on the fluorescence-activated cell sorter. We now present evidence that during normal B-lymphocyte differentiation V-C rearrangement occurs only on one chromosome.

In germ-line (sperm) and embryo DNA the variable and constant region genes coding for immunoglobulin heavy and/or light chains are on widely separated DNA segments, whereas in neoplastic plasma cell clones (myelomas) V- and C-region genes are found closer together. Thus, at some point during differentiation of plasma cells (or their B-cell precursors) V- and C-regions are rearranged to form a functional transcription unit. Allelic exclusion could therefore be the consequence of V- and C-region rearrangement on only one of the two chromosomes. The homologous chromosome would not undergo any change and therefore remain silent with respect to expression of the allelic immunoglobulin genes. Restriction fragment analysis of myeloma DNA has led to contradictory results concerning V-C rearrangements on homologous chromosomes: in some myelomas V-C rearrangement seems to have taken place on one chromosome only, as both new V-C gene arrangements and V- and C-containing DNA restriction fragments of the same size as in embryo DNA were found⁵. In other myelomas, only the newly arising restriction fragments carrying the rearranged V and C genes could be detected^{3,4} (R.J., unpublished observation for MOPC 167). This latter finding indicated that both homologous chromosomes might be involved in the translocation process that leads to a functional immunoglobulin gene. As most myelomas are aneuploid tumours carrying several chromosomal aberrations, it is not possible to use them as models for the study of the phenomenon of allelic exclusion.

Figure 1 shows a restriction map of the C κ gene in germ-line and embryo DNA⁶. The C κ gene is close to one end on a

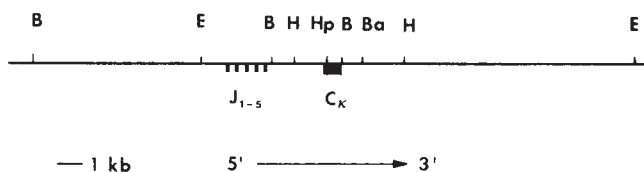


Fig. 1 Restriction map of the C κ gene in germ-line and embryo DNA⁶. The position of the J regions J₁₋₅ is according to Max *et al.*⁷ and Sakano *et al.*⁸. The arrow indicates the direction of transcription. The following restriction enzymes were used: *Bam*HI (Ba); *Bgl*II (B); *Eco*RI (E); *Hind*III (H); *Hpa*I (Hp). kb, kilobase.

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13-kilobase *Bam*HI fragment. Five J-region segments had been identified about 2.5–4.0 kilobases from the 5' end of the C_κ gene^{7,8}. Analysis of κ -chain clones from myeloma DNA had revealed that the 3' end of the V_κ to be expressed (including its 5'-flanking sequence⁹) was joined to the 5' end of a J region^{5,8}. Comparative analysis of restriction fragments of embryo and myeloma DNA resulted in restriction fragments of different sizes. The restriction enzyme *Bam*HI seemed to be a good choice for allowing V–J joining, because this event should always alter the 13-kilobase *Bam*HI fragment and create a V_κ - and C_κ -bearing *Bam*HI fragment of a different size. In fact, all κ -producing myelomas so analysed show new *Bam*HI fragments containing V_κ and C_κ sequences^{3–5}. Each new restriction fragment created by V–C translocation could differ in size from one lymphocyte to the other depending on which variable region has joined with which J region. As there are a large number of germ-line V_κ genes, *Bam*HI restriction fragment analysis of κ -light chain-producing cells does not necessarily show any particular new fragments, because the specific rearrangement of each lymphocyte would not be detected in a heterogeneous population of κ -bearing B cells. If V–C region rearrangement involved both homologous chromosomes, a disappearance of the 13-kilobase *Bam*HI germ-line fragment should be seen. However, if this event occurs on one chromosome only, the 13-kilobase *Bam*HI fragment should be retained.

We isolated splenic B lymphocytes having surface κ light chain. Staining of spleen cells with rabbit anti- κ immunoglobulin (Ig) and fluorescent goat anti-rabbit Ig antibodies yielded two different populations of cells. About 50–55% of spleen cells gave positive fluorescence when stained with a

rabbit anti- κ Ig whereas the remaining 45–50% did not stain above background (controls for background were the same cells stained at the same concentration with rabbit anti-keyhole limpet haemocyanin antibodies and the fluorescent goat anti-rabbit Ig). These two populations were purified on the fluorescence-activated cell sorter¹⁰. We isolated κ -positive (κ^+) from κ -negative (κ^-) cells. To minimise contamination, cells weakly positive for κ were not sorted as κ^+ cells. Contamination of sorted κ^+ with κ^- cells was less than 4%. DNA of κ^+ cells were purified, digested with *Bam*HI and electrophoresed through agarose to separate the resulting restriction fragments. After transfer of the DNA to DBM paper¹¹, restriction fragments bearing κ -region genes were detected by hybridisation with a nick-translated plasmid containing a purified C_κ -region insert⁶.

Table 1 Relative band intensities of various DNA digests

DNA digest	Relative band intensity	
	C_κ	Ribosomal
5 μ g <i>Bam</i> HI sperm	85	245
10 μ g <i>Bam</i> HI sperm	172	561
10 μ g <i>Bam</i> HI κ^+	87	664

The relative band intensities were determined by densitometry. Analysis of *Bam*HI-digested DNA with CD18 was used as internal standard and the intensities of all bands per lane were added up.

Figure 2 shows the results of this experiment. DNA from κ^+ cells is compared with different amounts of sperm DNA. The 13-kilobase *Bam*HI germ-line fragment is clearly detectable in κ^+ DNA. We interpret this to demonstrate that both homologous chromosomes have not undergone a rearrangement with respect to their V_κ and C_κ genes. If V–J joining takes place on one chromosome one would expect a 13-kilobase *Bam*HI fragment of half the intensity compared with the same amount of germ-line (sperm) DNA. To control for possible inaccuracies in the determination of the input DNA amounts, the band intensities were compared with an internal standard. For this, the DBM paper was washed in 0.4 M NaOH for 30 min, neutralised and rehybridised to a ³²P-labelled plasmid containing a ribosomal DNA insert¹². These results are shown in Fig. 2 and summarised in Table 1. The intensity of the ribosomal bands of 10 μ g sperm and 10 μ g κ^+ DNA after *Bam*HI restriction is the same. However, the intensity of the 13-kilobase C_κ fragment of 10 μ g κ^+ DNA corresponds to that of the 5 μ g sperm DNA. This is in agreement with the idea that V–C joining occurs on one chromosome during differentiation of κ -chain-expressing B lymphocytes. These experiments clearly demonstrate that in the majority of B cells V–J joining and therefore the formation of a functional transcription unit for κ chains does not occur on both chromosomes. However, a small fraction of B cells could have undergone V–J joining on both chromosomes and would not have been detected by this assay. We speculate that the consequence of V–J joining on one chromosome is the allelic exclusion of immunoglobulin gene products in B cells and plasma cells. Given that allelic exclusion may result from differential V–J joining, one must consider potential molecular mechanisms for this event. A reasonable assumption is that differentiating lymphoid cells of the B-cell lineage contain some highly specific enzymes that accomplish the V–J joining. It is not clear how these putative recombinases (or other enzymes effecting V–J joining) use only one of two identical chromosomes as their substrate and leave the second (homologous) one unchanged. Several possible mechanisms could lead to the activation of immunoglobulin genes on one chromosome only. One can imagine the production of one essential enzyme molecule (either the recombinase or a molecule that renders the chromosome susceptible to recombinase action) per cell that works in a stoichiometric fashion—one molecule per chromosome. Once V–J joining has been initiated the enzyme molecule is no longer active. Differentiating lymphoid cells that do not produce this enzyme would not enter the B-cell lineage.

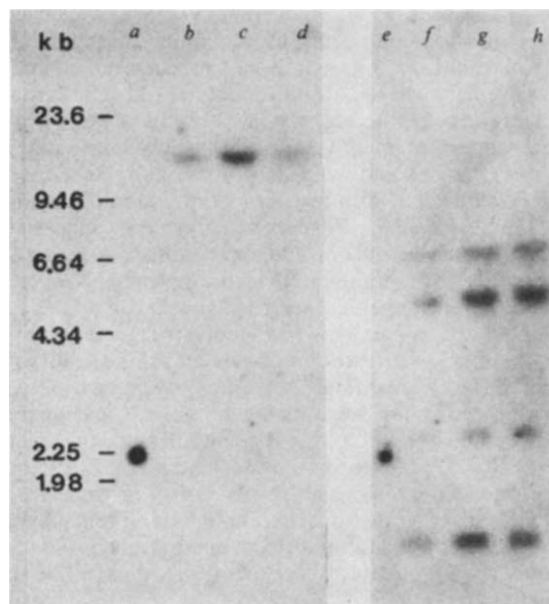


Fig. 2 A spleen cell suspension from BALB/c mice was treated on ice with 10 vol 8.3 g l⁻¹ NH₄Cl and 1 g l⁻¹ KHCO₃, pH 7.4, to lyse erythrocytes. The nucleated cells were collected by centrifugation, washed, stained with 40 μ g ml⁻¹ rabbit anti-mouse κ antibodies and counterstained with 75 μ g ml⁻¹ fluorescent goat anti-rabbit Ig antibodies. About 55% of the cells stained with the anti- κ antibodies. The brighter 50% of the spleen cells were sorted on a fluorescence-activated cell sorter⁹ (FACS III, Becton Dickinson). On re-analysis, this population was about 96% κ^+ . DNA was purified and analysed with *Bam*HI as described elsewhere⁶. The restriction fragments were electrophoresed through 0.7% agarose and transferred to DBM-paper¹⁰. Hybridisation was to ³²P-labelled C_κ probe (lanes a–d) or a plasmid containing a *Xenopus laevis* ribosomal DNA insert (CD18)¹¹ (lanes e–h). a, e, 1 μ g sperm DNA; b, f, 5 μ g sperm DNA; c, g, 10 μ g sperm DNA; d, h, 10 μ g κ^+ DNA. The molecular weight standard is *Hind*III-digested λ DNA.

A slightly different possibility is that a limiting amount of the enzyme is produced. If the enzyme level is too low no V-J joining occurs, and these cells will not become B lymphocytes. At higher enzyme levels one chromosome can be activated, leading to immunoglobulin-producing cells. At still higher levels (or infrequently at low enzyme levels) one would expect a rearrangement of V and C genes on both chromosomes in a small subpopulation of differentiating cells. This could lead to death of those cells if the V-C interval contains gene(s) essential to cell viability, and if this interval is excised during differentiation. A third possibility would be that as soon as rearrangement has been initiated a negative feedback prevents new rearrangements from taking place. Whatever the situation, selective chromosomal expression in the case of the κ chain accompanies selective chromosomal rearrangements. In

contrast, X-chromosomal allelic exclusion is apparently the consequence of selective chromosomal inactivation¹³. Nevertheless, it is possible that both activation and inactivation of chromosomal segments may result from selective irreversible chromosomal rearrangements.

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Evidence for the involvement of H1 histone phosphorylation in chromosome condensation

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Changes in the structural organisation of chromatin are necessary for the progression of the cell cycle. These changes are thought to be regulated mainly by the modification of chromosomal proteins by reactions such as phosphorylation¹⁻⁸, acetylation⁹⁻¹², methylation^{13,14} and poly(ADP-ribosylation)^{15,16}. Among these modifications, phosphorylation of histones, especially that of the H1 histone, is the most likely candidate for a factor which regulates chromosome condensation¹⁻⁸. It has been proposed that the phosphorylated form of H1 histone may be involved in the initiation of chromosome condensation¹⁻⁴ or in the maintenance of the condensed state of chromatin⁸. The latter possibility is unlikely because zinc chloride, which inhibits phosphatase *in vivo* and preserves the highly phosphorylated form of H1 histone at metaphase, does not prevent the dispersion of chromosomes at the end of mitosis¹⁷. As for the former possibility, Bradbury *et al.* reported² that the activity of H1 histone phosphorylation is closely correlated with mitotic triggering in the cell cycle and suggest^{3,4} that H1 histone phosphokinase is involved in the initiation of mitosis. However, no causal relationship has been established. To analyse the mechanism of the progression of the cell cycle, we have isolated several temperature-sensitive (ts) growth mutants from FM3A, a cell line derived from C3H mouse mammary carcinoma¹⁸. We report here on one of these ts mutants, designated the ts85 strain, which shows an abnormal chromosome condensation as well as deficiency in H1 histone phosphorylation at the non-permissive temperature. Our results support the hypothesis that H1 histone phosphorylation controls the initiation step of mitosis through chromosome condensation.

The ts85 cells were obtained as follows. Logarithmically growing FM3A cells in suspension culture were treated with 0.3 $\mu\text{g ml}^{-1}$ N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 16 h at 33 °C and incubated for 3 days of expression time. Wild FM3A cells were killed by the addition of ³H-thymidine (15 Ci mmol⁻¹ 5 $\mu\text{Ci ml}^{-1}$) for 16 h after the cells had been shifted up to 39 °C, the non-permissive temperature for ts cells. The selection schedule was repeated several times and then the surviving cells were inoculated into soft agar plates^{19,20} and incubated at 33 °C (permissive temperature) for about 2 weeks. The colonies which developed were checked for growth at 33 °C and 39 °C. The ts85 was one of the clones which showed no increase in the cell number at 39 °C but normal growth at 33 °C.

The ts85 cells were synchronised at the G1/S boundary by double treatment with excess thymidine at 33 °C and then permitted to enter the S phase at 33 °C or 39 °C. The amount of DNA synthesised at 39 °C was almost the same as that at 33 °C, but no mitotic cells were detected at 39 °C (Fig. 1), indicating that at the non-permissive temperature ts85 cells were arrested before the mitotic phase. When ts85 cells growing logarithmically at 33 °C were shifted up to 39 °C and incubated for 16 h, using a cytofluorometer, most of the cells were found to be arrested at the G2 phase. Details of the growth characteristics of ts85 will be reported elsewhere²¹.

To determine whether or not ts85 cells contain condensed chromatin at the arrested state at 39 °C, electron microscopic observations were carried out. As shown in Fig. 2, a small fraction of the chromatin was condensed into a fragmented form (arrow in Fig. 2), but the structures of the nuclear membrane and nucleoli were preserved. The incomplete condensation was observed in 80% of the total population after incubation for 16 h at 39 °C. Thus, most of the chromatin did not initiate condensation at the arrested state and as a result, the cells were not able to enter the mitotic phase.

If H1 histone phosphorylation stimulates the initiation of chromosome condensation¹⁻⁸, cells with a defect in chromosome condensation might have a deficiency in H1 histone phosphorylation. This is the case with ts85 cells. These cells were synchronised at the G1/S boundary, released from the blockade, incubated at 33 °C or 39 °C and the phosphorylation pattern of the histones was examined. Figure 3 shows the incorporation of ³²P and the densitometric profile of histones isolated from G2, M phase cells and cells arrested at 39 °C. The rate of phosphorylation at 39 °C of the H1 histone decreased to 1/10th of that at 33 °C (Fig. 3). The rates of phosphorylation of H2A and H4 histones, however, were somewhat higher at 39 °C

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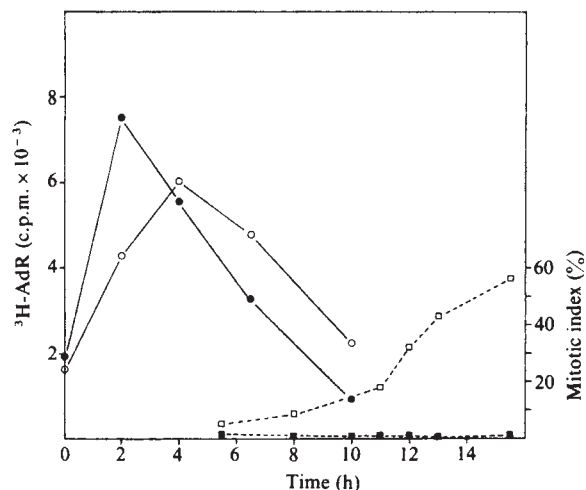


Fig. 1 DNA synthesis and entry into mitosis after synchronisation at the G1/S boundary. Logarithmically growing ts85 cells in suspension were treated with excess thymidine (2 mM) in RPMI-1640 containing 10% dialysed calf serum at 33 °C for 18 h. After removing excess thymidine, cells were incubated at 33 °C for 8 h in normal medium, then the second treatment with excess thymidine for 10 h at 33 °C was given. The ts85 cells which accumulated at the G1/S boundary were permitted to enter the S phase at 33 °C or 39 °C in normal medium containing 0.03 $\mu\text{g ml}^{-1}$ colcemid (Ciba). DNA synthetic activity at 33 °C (○) or 39 °C (●) was measured by pulse labelling with ³H-deoxyadenosine (AdR, 1 $\mu\text{Ci ml}^{-1}$, 21 Ci mmol⁻¹) for 30 min at each temperature. The alkaline-resistant radioactivity in acid-insoluble materials collected on Whatman glass fibre filters (type GF/C) was measured by liquid scintillation spectrometer. Mitotic index at 33 °C (□) or 39 °C (■) was measured by microscopic observation of cells treated with 1% sodium citrate and fixed with methanol/acetic acid (3:1 v/v).

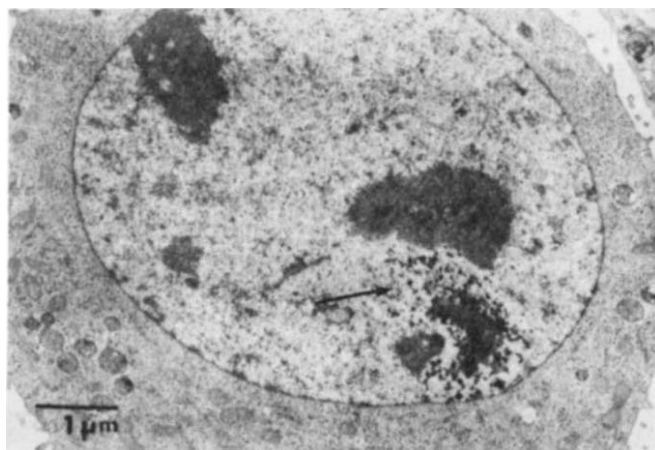
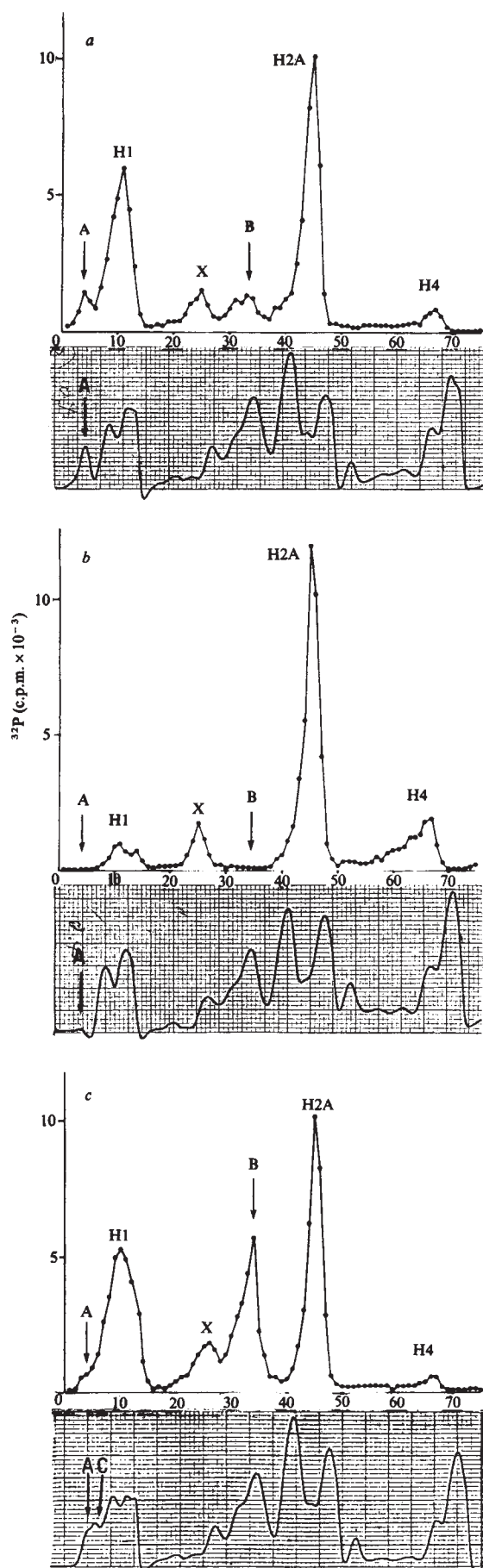


Fig. 2 Electron microscopic observation of a ts85 cell possessing fragmented abnormal condensation of chromatin (indicated by arrow) in the nucleus at 39 °C. Logarithmically growing ts85 cells at 33 °C were incubated at 39 °C for 16 h. After incubation, the cells were washed with 0.1 M phosphate buffer (pH 7.4) and fixed with phosphate-buffered 2.5% glutaraldehyde (pH 7.4) for 1 h at room temperature. After a wash with the same buffer, cells were post-fixed with 1% osmium tetroxide in the same buffer for 1 h at room temperature. The fixed cells were embedded in Epon-Araldite, cut into thin sections using a Sorvall ultramicrotome and observed with a JEOL 100B transmission electron microscope operated at 80 kV. In wild-type cells at both temperatures and ts85 cells at 33 °C, no such fragmented abnormal condensation of chromatin was detected throughout their cell cycles.

Fig. 3 The profile of phosphorylation of histones of ts85 cells at the permissive and non-permissive temperatures. The ts85 cells were synchronised at the G1/S boundary as described in Fig. 1 legend. When the cells were released from the blockade and entered the S phase, they were pulse labelled with ^{32}P -orthophosphate ($100\ \mu\text{Ci ml}^{-1}$, carrier free) for 2 h at 6 and 13.5 h after release from the blockade, corresponding to the G2 and M phases at 33°C . The ts85 cells arrested at the G2 phase at 39°C were labelled at 13.5 h. Histone fractions were extracted as follows. Cells frozen at -20°C ($5\text{--}10 \times 10^6$ cells) were thawed and suspended in 1 ml of hypotonic buffer T (10 mM Tris-HCl, pH 7.8, 2 mM MgCl_2 , 0.5 mM EDTA, 2 mM 2-mercaptoethanol and 0.025% Triton X-100). After standing for 10 min at 4°C , they were homogenised by 15 strokes with a Teflon pestle glass Potter Elvehjem homogeniser and centrifuged at $800g$ for 5 min. The precipitate was washed successively with buffer T, buffer A (50 mM Tris-HCl, pH 7.4, 0.14 M 2-mercaptoethanol and 50 mM sodium bisulphite) and buffer A containing 0.15 M NaCl, with stirring. Nuclei were pelleted at $12,000g$ for 10 min. Histones were then extracted by 0.4 N sulphuric acid for 1 h with stirring at 4°C . Histones obtained were pelleted by 18% trichloroacetic acid and washed once with acetone-HCl (HCl 0.5% v/v) and twice with acetone. They were analysed by acid urea 15% polyacrylamide gel electrophoresis (0.9 N acetic acid–2 M urea, 25 cm gel) according to the method of Panyim and Chalkley²³. After electrophoresis for 30 h, the gels were stained with Coomassie brilliant blue R and were scanned with a densitometer. Gels were frozen at -80°C and cut into 1.2-mm slices. Each slice was put into a vial, solubilised with Soluene 350 (Packard) and the radioactivity measured by a liquid scintillation spectrometer using toluene-based scintillation fluid. a, G2 phase at 33°C ; b, G2-arrested at 39°C ; c, M phase at 33°C . A densitogram is shown under the profile of each phosphorylation pattern. X was the unknown protein which existed in wild-type cells and ts85 cells at both temperatures. The peaks which appeared in the densitogram, from left to right, correspond to H1, H3, H2B, H2A and H4 histone, respectively.

than at 33°C . The highly phosphorylated form of the H1 histone which was detected in M phase cells (indicated by arrow C in Fig. 3c) did not exist in the G2-arrested cells at 39°C . It has been reported that when the synthesis of histones is blocked by an inhibitor of DNA synthesis⁷ or X-ray irradiation²², the phosphorylation of all the histones is apparently reduced. In the ts85 cells, the amount of histones synthesised at 39°C was 85% of that at 33°C and the synthesis of H1 histone was not specifically inhibited at 39°C . Furthermore, a band behind the H1 histone subfractions (as shown by arrow A in Fig. 3), was found at 33°C but not at 39°C . This protein is insoluble in 5% perchloric acid (PCA) and extracted by 0.2 M sulphuric acid. The role of this protein is unknown but it may be related to chromosome condensation. The specific decrease in the rate of H1 histone phosphorylation and the absence of the highly phosphorylated form of H1 histone at 39°C did not result from cessation of the cell cycle at the G2 phase, because the rate of H1 histone phosphorylation remained constant throughout the S to M phases in both wild-type cells and grow-revertant ts85 cells. Other ts mutants (ts18 of FM3A cells, 11C3 of CHO cells) which were also arrested at the G2 phase at 39°C had no defects in H1 histone phosphorylation (unpublished data).

Figure 3 also shows that H3 histone (indicated by arrow B) was not phosphorylated at 39°C , whereas at 33°C ts85 cells entered the mitotic phase and marked H3 histone phosphorylation occurred^{5,7,8}. This lack of H3 histone phosphorylation may be due to the cessation of the cell cycle, that is, ts85 cells may have been arrested before the onset of the mitotic phase.

The above data suggest that the phosphorylation of H1 histones is one of the factors involved in the initiation of chromosome condensation and the low level of H1 histone phosphorylation may induce incomplete condensation of the chromatin.

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The relationship between coding sequences and function in haemoglobin

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Gilbert has suggested that the presence of intervening sequences in DNA, called introns, can speed evolution by allowing novel proteins to be constructed from the pieces of existing ones¹. This hypothesis further suggests that the coding sequences, called exons, correspond to functional parts of the protein. The most striking example so far is the case of the immunoglobulin γ heavy chain, where the four polypeptide sequences corresponding to the four coding sequences form structurally and functionally distinct parts of the molecule^{1,2}. The relation between the three coding sequences of the β globin gene and structure or function is not as obvious, but the central coding sequence does code for the part of the globin chain which forms the haem crevice^{3,4}. To further test the idea that coding sequences correspond to functional units of proteins we consider the relationship between the coding sequences of α and β globin genes and the corresponding parts of the complete, tetrameric haemoglobin molecule.

The principal function of haemoglobin in vertebrates is to transport oxygen. Haemoglobin performs this function effectively by binding oxygen cooperatively, and with an overall affinity appropriate to physiological conditions. The affinity is regulated by protons and carbon dioxide (the Bohr effect), and by organic phosphates (2,3-diphosphoglycerate (DPG) in mammals). According to the simplest allosteric model for cooperative behaviour⁵, haemoglobin exists in two affinity states, corresponding to the quaternary structures of oxyhaemoglobin (R state) and deoxyhaemoglobin (T state). Binding within either the T or R state is noncooperative. Cooperativity is achieved by switching from the low affinity T state to the high affinity R state as successive molecules of oxygen bind. Regulation of oxygen affinity is achieved by the preferential binding of protons, carbon dioxide, and organic phosphates to the low affinity T state. The difference in tertiary conformation within

each quaternary structure produces a difference in affinity of the haems for oxygen. The model is somewhat oversimplified, but provides a good first order description of functional properties and a useful framework for correlating structure with function⁶⁻¹¹.

The large number of investigations on structure-function relationships in haemoglobin, which were pioneered by the work of Perutz and coworkers, make it possible to assign well defined functional roles to individual amino acid residues^{6,12,13}. From inspection of the three-dimensional structure of human haemoglobin, five functional categories are apparent^{6,11,14-16}. (1) Residues in contact with the haem. These residues provide the hydrophobic environment necessary for the haem to be oxygenated by oxygen, rather than be oxidised. Haem contact residues also transmit structural changes, caused by oxygenation at the haem, to the $\alpha_1\beta_2$ subunit interface. (2) Residues in the $\alpha_1\beta_1$ contact. When haemoglobin switches quaternary structure, the $\alpha_1\beta_2$ intersubunit bonding is altered, but the same residues are involved in both quaternary structures. (3) Residues in the $\alpha_1\beta_1$ contact. This contact remains unaltered by the quaternary structure change. (4) Residues of the β chain that bind DPG. (5) Residues involved in the Bohr effect. As protons are not resolvable in the X-ray electron density map of haemoglobin, these residue assignments require detailed consideration of both the structure and solution chemistry^{6,11,17}.

Functional studies on haemoglobin variants provide support for the residue assignments. The functional residues, together with the known variants of human haemoglobin^{18,19} are listed in

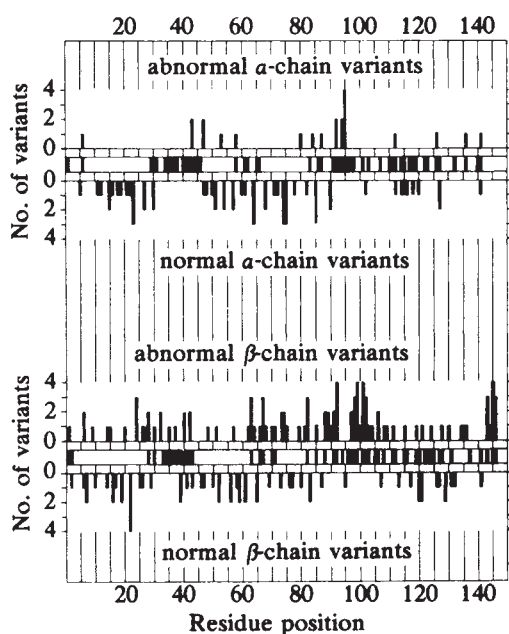


Fig. 1 Residues with well defined functional roles in human haemoglobin and α and β variants. For each chain, positions are marked for those residues that belong to one of the five categories discussed in the text. Haem contact, $\alpha_1\beta_2$ contact, and $\alpha_1\beta_1$ contact residues for deoxyhaemoglobin are taken from Fermi¹⁴. Haem contact, $\alpha_1\beta_2$ contact and $\alpha_1\beta_1$ contact residues for human carbomonoxyhaemoglobin, defined as a residue containing at least one atom within 4 Å of a haem atom or adjacent subunit atom, were calculated from the coordinates deposited by Joyce Baldwin with the Brookhaven Protein Data Bank. DPG binding site residues are taken from Arnone¹⁶, and Bohr effect residues are taken from Baldwin and Chothia¹¹ and Matthew *et al.*¹⁷. Carbon dioxide binds to α -amino groups of both α and β chains. Above the functional residues list are the number of variants at each residue position with known abnormal properties *in vitro*, and below the functional list are the number of variants at each residue position with no known abnormal properties *in vitro*. For the most part, the variants have not been studied in great detail. It is, therefore, likely that some variants listed as normal may exhibit functional impairment when examined further. The list of haemoglobin variants is published in the journal *Hemoglobin*^{18,19}.

Fig. 1. Among α chain variants with known abnormal properties *in vitro*, 77% (17 out of 22) contain substitutions at one of the functional residue positions, while 83% (48 out of 58) of the variants with no known abnormal properties *in vitro* contain substitutions at 'non-functional' residue positions. Among β chain variants the corresponding values are 63% (66 out of 104) and 83% (49 out of 59). If clinical findings are used as criteria, all four percentages are higher^{12,29}. These statistics support the relative significance of the residues in the five categories, but also suggest that functionally significant residues are missing from the list. For example, there are additional residues in the interior of the protein that maintain the stability of the three-dimensional structure, and additional residues that undergo conformational changes as part of the cooperative binding mechanism^{11,20}. Also, residues on the haemoglobin surface function to prevent aggregation of tetramers (as occurs in sickle cell disease) at the high concentrations found in the red cell²¹. Residue assignments for these functions cannot at present be made with confidence.

The residues associated with each of the five functions are listed in Fig. 2, which also shows their distribution in relation to the three coding sequences of the α and β globin genes. First, as pointed out by Blake³ and Gibert⁴, most of the residues (16 out of 20) in contact with the haem correspond to the central coding sequence of the β globin gene. In the α gene, 16 out of 20 of the haem contact residues are also contained in the central sequence. The distribution of intersubunit contacts among the three coding sequences shows a very strong correlation as well. In the case of the $\alpha_1\beta_2$ contact, the central sequence contains 12 out of the 15 residues in the β subunit that form intersubunit bonds, and 13 out of 18 residues in the α subunit. For the $\alpha_1\beta_1$ contact the third sequence contains 13 out of the 18 intersubunit bonding residues in the α subunit, and 14 out of 18 in the β subunit. The distribution of residues involved in binding the regulators—protons, carbon dioxide, and DPG—indicates no marked correlation, although the most important residues are contained in the first and third sequences.

The distribution of functional residues among the three coding sequences suggests that coding sequences do correspond to the major functions of haemoglobin. It is therefore interesting to speculate on the possible role that coding sequences played in the evolution of haemoglobin. One possible evolutionary pathway for mammalian haemoglobin is the following. The primitive haemoglobin is assumed to be a monomeric protein, capable of reversible oxygen binding, and coded for by a gene containing three separate coding sequences. Following gene duplication point mutations in the central coding sequence of the primitive α and β globin genes led to the development of the $\alpha_1\beta_2$ dimer that was capable of cooperative oxygen binding. (Note that lamprey haemoglobin, which has previously been considered to behave as a possible intermediate in the evolution of haemoglobin tetramers from monomers^{26,27}, is a monomer when oxygenated. It achieves cooperativity by aggregating in the deoxy state to form a homodimer, which is very probably of the $\alpha_1\beta_2$ type^{26,27}.) The maximum Hill n value for such a dimer was 2. An increase in cooperativity toward the current n value of 2.8 to 3.0 took place with the evolution of the $\alpha_1\beta_1$ contact to form a tetramer. The concentration of $\alpha_1\beta_1$ contact residues in the third sequence suggests that the third coding sequence in the α or β globin gene (or both) replaced its predecessor by recombination, thereby speeding the evolutionary process. The most recent events of evolution involved development of the Bohr effect and DPG binding residues, which are responsible for fine-tuning haemoglobin function. The almost complete absence of haem or intersubunit contacts in the first polypeptide sequence makes it conceivable that the corresponding coding sequence also replaced the preceding one in both α and β globin genes by recombination, thus more rapidly completing the evolution of the regulation of oxygen binding by protons, carbon dioxide, and DPG.

Considerations of haemoglobin evolution from amino acid sequence comparisons among species do not appear to argue

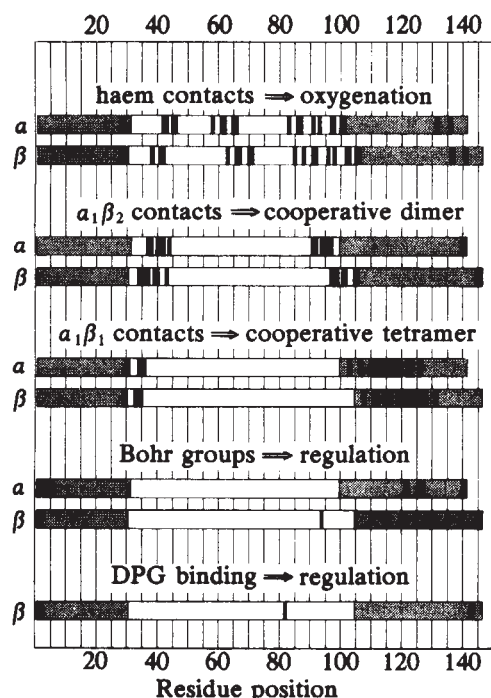


Fig. 2 Residues with well defined functional roles in human haemoglobin and the coding sequences of the mouse α and β globin genes. In the mouse α globin gene, the three coding sequences correspond to the amino acid residue positions 1–31, 32–99, and 100–141, (refs 22, 23) while in mouse β globin the sequences are 1–30, 31–104, and 105–146, which are structurally homologous to the α globin sequences^{24,25}.

strongly for or against this pathway in which the current first and third coding sequences arose from replacement of whole sequences in single steps. Goodman *et al.* have calculated that the number of nucleotide replacements per amino acid, from the point of gene duplication to the α and β chain amniote ancestors, was greater for the $\alpha_1\beta_2$ contact residues than for either the haem contact or $\alpha_1\beta_1$ contact residues¹⁵. In contrast, during the subsequent evolution to the current haemoglobins, the number of nucleotide replacements per position was greater for the $\alpha_1\beta_1$ contact residues than for either the haem or $\alpha_1\beta_2$ contact residues. If one takes the point of view that amino acid replacements signal the evolution of function at those positions, then these data support the idea that in the $\alpha_1\beta_2$ contacts evolved before the $\alpha_1\beta_1$ contacts.

The observation of a correlation between coding sequences and function presented here suggests that an investigation of the functional properties of the three polypeptide sequences of haemoglobin corresponding to the three coding sequences could provide new insight into gene–function relationships. Preparation of several of the sequences by digestion with trypsin is possible²⁸, since arginines are strategically located in both α and β chains, and in some haemoglobin variants^{18,19}.

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Isolation of chromosomal origins of replication in yeast

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Origins of replication have been identified in the DNA of viruses¹, mitochondria², bacterial plasmids³ and the bacterial chromosome⁴. However, origins of replication of eukaryote chromosomes have remained elusive because of the large size and sequence complexity of chromosomes and in particular for want of a suitable assay for their detection. Recent development of techniques for genetic transformation of yeast by autonomously replicating cytoplasmic plasmids^{5–8} now makes it possible to search for eukaryote origins in a manner analogous to that used for bacteria⁴. Here we describe the construction and properties of a plasmid which contains no effective eukaryote replication origin and whose efficiency of replication in yeast is greatly enhanced by insertion of certain fragments of yeast chromosomal DNA. We believe these to contain replication origins, since yeast transformants are shown to contain copies of the transforming plasmids.

The 13.7-kilobase recombinant plasmid pJDB248, consisting of the yeast 2 μ m plasmid, of pMB9 (a derivative of ColEI) and a 2.4 kilobase fragment of yeast DNA containing the *LEU2* gene, can be used to transform *Saccharomyces cerevisiae* M16, *leu2-3, his4-712^{FS}, ade2-1, lys2-1, SUF2*, to the *leu*⁺ phenotype with a frequency of 10^4 – 10^5 transformants per μ g of DNA⁵. To obtain a plasmid whose presence in yeast can be detected, yet which lacks the origin of replication provided by the 2 μ m plasmid, a 1.5 kilobase *HaeII* fragment of pJDB248 containing the *LEU2* gene was recombined into the tetracycline resistance gene of the bacterial plasmid pBR325 (Fig. 1). The resultant 6.2-kilobase molecule, pDAM1, can transform a leucine-requiring strain of *Escherichia coli* (JA221) to *leu*⁺ and to chloramphenicol resistance with equal efficiency.

However, the ability of pDAM1 to transform yeast (M16) to *leu*⁺ is much impaired compared with pJDB248. pDAM1 gives rise to two clearly distinguishable types of yeast transformant (Table 1). One grows rapidly on leucine-free medium (1–10 transformants per μ g DNA) and probably results from recombination of pDAM1 or part of it with host chromosome¹⁰. The other is much more frequent (20,000 transformants per μ g DNA) but grows extremely slowly, the cells taking up to five days to form microcolonies visible to the naked eye. Cells from these colonies can be serially recombined on leucine-free medium, giving rise to further microcolonies, although the relative cloning efficiency is only 0.01–0.1% of that in the presence of leucine (Table 1). By contrast, pJDB248 transformants have a relative cloning efficiency of 78%. Furthermore, these pDAM1 transformants produce no visible turbidity after inoculation of a 2 ml leucine-free liquid culture even after a week. These data are taken to indicate that pDAM1 can survive and express the

LEU2 gene in yeast, but that the rate of growth of these transformants is limited by the very low copy number of the plasmid, perhaps no more than one molecule per 1,000–10,000 cells, resulting from the failure of pDAM1 to replicate.

It is possible to insert fragments of yeast DNA into pDAM1 and screen for those which give rise to yeast transformants which grow rapidly in the absence of leucine. Yeast whole cell DNA was digested with *Pst*I, ligated with the *Pst*I site within the ampicillin gene of pDAM1, and the total mixture cloned in *E. coli*. DNA preparations were made from 107 separate bacterial colonies and each was tested for its ability to transform yeast to *leu*⁺. Of such preparations 99 gave rise to microcolony transformants, indistinguishable in growth properties from those of pDAM1 itself, whereas 8 (pDAMY1 to 8) gave rise to colonies all of which grew rapidly, similarly to pJDB248 transformants (Table 1). Analysis by restriction digestion of these 8 DNA preparations showed two—pDAMY5 and pDAMY6—to contain the full length yeast 2 μ m plasmid which is endogenous to the strain (M16) from which the yeast DNA was prepared, and has one *Pst*I restriction site. The remaining 6 have *Pst*I inserts into pDAM1 ranging in size from 0.7 to 6.3 kilobases which are presumed to derive from the yeast chromosomal DNA (Table 1).

To determine whether copies of these plasmids are present in yeast transformants, lysates of single yeast colonies were prepared and the DNA analysed by agarose gel electrophoresis. The DNA was transferred to nitrocellulose filters and hybridised to ³²P-labelled pBR325 (Fig. 2). Tracks 1 and 2 of the autoradiograph contain DNA from the untransformed host strain (M16) and a low frequency transformant of pDAM1. Neither of these shows hybridisation to pBR325 detectable in this experiment. A high frequency transformant of pDAM1 was not tested as these colonies do not grow adequately on leucine-free media. However, transformants of pDAMY1, pDAMY2 and pJDB248 (tracks 3–8) have bands, hybridising to pBR325, with electrophoretic mobilities corresponding to the supercoiled (rapidly migrating) and open-circular (slower migrating) forms of the transforming plasmids. The presence of plasmids hybridising to pBR325 has been found in transformants of each of pDAMY1–8 (data not shown).

Why does insertion of certain fragments of yeast DNA into pDAM1 greatly increase the ability of transformants of this

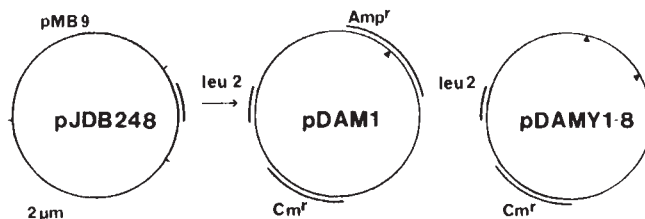


Fig. 1 Illustration of the relationship between the plasmids pJDB248, pDAM1 and pDAMY1–8. pJDB248 consists of pMB9, the yeast 2 μ m plasmid and a 2.4-kilobase insert complementing the yeast *leu2* mutation. pDAM1 consists of pBR325 with a 1.5-kilobase insert containing the *LEU2* gene. This plasmid also carries chloramphenicol resistance (*Cm*^r) and ampicillin resistance (*Amp*^r). pDAMY1–8 are derived from pDAM1 by insertion of *Pst*I fragments of yeast DNA into the *Pst*I site (Δ) of the ampicillin gene of pDAM1. To construct pDAM1, pBR325 purified by the cleared lysate procedure⁹ and caesium chloride centrifugation was partially digested with the restriction endonuclease *Hae*II and ligated with a partial digest of pJDB248. The ligated DNA was used to transform *E. coli* JA221 *recA1*, *leuB6*, *trp* Δ *ES*, *hsdR*[–]*hsdM*⁺ *lacY* C600, to *leu*⁺ using minimal selective plates. pDAM1 was one such transformant, consisting of a 1.5-kilobase *Hae*II fragment containing the *LEU2* gene inserted into the tetracycline resistance gene of pBR325. Approximately 0.75 kilobases of this gene are absent. Yeast DNA was prepared from the strain M16 by lysis of protoplasts⁵, phenol extraction and ethanol precipitation. The DNA was digested with *Pst*I and ligated with a *Pst*I digest of pDAM1. This DNA was used to transform JA221 to chloramphenicol resistance and ampicillin sensitive colonies containing inserts of yeast DNA were subsequently selected.

plasmid to grow in the absence of leucine? It is most unlikely that this phenomenon is related to the efficiency of expression of the *LEU2* gene. This gene derived from yeast and carried in pDAM1 is expressed adequately in *E. coli*. Equally, it is unlikely that the result is a consequence of the ability of certain fragments of yeast DNA to allow vastly more efficient integration of pDAM1 into the yeast chromosome. Such integration may occur but it does not readily account for the presence of free plasmid copies in transformants. We believe that the poor growth of pDAM1 transformants is due to a very low copy

Table 1 Properties of pDAM1, its derivatives, and the yeast transformants which they produce

Plasmid	Size (kilobases)	Transformation frequency colonies per μ g DNA	Relative cloning efficiency <i>leu</i> [–] / <i>leu</i> ⁺ (%)
pDAM1	6.2 A	1–10	98
	B	20,000	0.01–0.1
pDAMY1	10.5	10,000	32
pDAMY2	11.2	10,000	50
pDAMY3	12.5	10,000	25
pDAMY4	9.1	10,000	65
pDAMY5	12.0	10,000	12
pDAMY6	12.0	10,000	8
pDAMY7	12.4	10,000	9
pDAMY8	6.9	10,000	2

The plasmids pDAMY1 to 8 are derived from pDAM1 as described in the legend to Fig. 1. Their size is given in kilobases and the approximate frequencies with which they transform the yeast strain M16 to *leu*⁺ are also given. The methods for yeast transformation was as previously described⁵. The relative cloning efficiency of yeast transformants was obtained as follows. Transformant colonies were spread on leucine-free minimal plates and allowed to grow overnight at 29 °C. Approximately 1,000 cells were suspended in 0.5 ml H₂O, aliquots of 100 μ l were spread onto leucine-containing and leucine-free minimal plates. Colonies were counted after 2 days except that pDAM1 microcolonies required 5 days to grow. The relative cloning efficiency is the ratio of the number of colonies on *leu*[–] plates to that on *leu*⁺ plates expressed as a percentage.

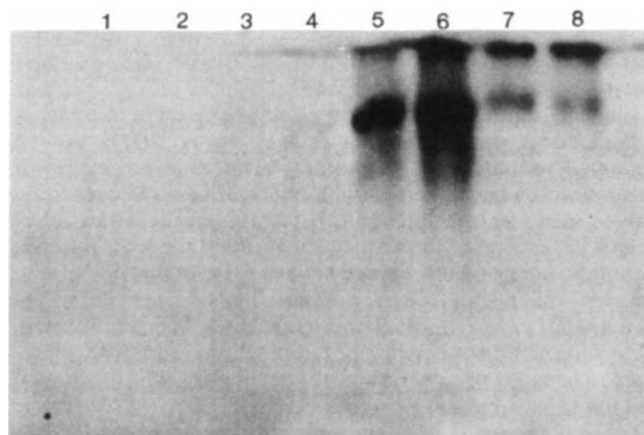


Fig. 2 Autoradiograph of ³²P-labelled pBR325 hybridised to undigested DNA of yeast transformants. Track 1: untransformed recipient strain M16. Track 2: rapidly growing transformant of pDAM1. Tracks 3,4: transformants of pDAMY1. Tracks 5,6: transformants of pDAMY2. Tracks 7,8: transformants of pJDB248. DNA was prepared from transformant colonies⁵, which had been grown on leucine-free media, and electrophoresed through 0.8% Agarose at 3 V cm^{–1} for 15 h. DNA was transferred to a nitrocellulose filter¹⁰ and hybridised with 4 $\times$ 10⁶ d.p.m. ³²P-labelled pBR325 prepared by nick translation. The filter was exposed to Fuji Rx film with a Kodak intensifying screen at –60 °C for 4 days.

number of the molecule resulting from a lack of a eukaryotic origin of replication. The little replication that certainly does occur may be due to utilisation of the bacterial origin contained in pBR235. Those derivatives of pDAM1 which allow rapid growth of transformants are thought to be present in a higher copy number than pDAM1 because they contain origins of replication. This view allows an estimation of the frequency of origins in the yeast chromosome. Of 105 *Pst*I fragments of yeast DNA tested (excluding those two shown to be the 2 μ m plasmid), 6 contain origins. As the average size of the *Pst*I inserts in our clones is 2.3 kilobases there is, on average, one origin in every 40.2 kilobases of chromosome. This compares with the figures of 68 and 90 kilobases obtained respectively by electron

microscopy and fibre autoradiography of replicating yeast DNA^{12,13}. It remains to be established whether the apparent origins of replication detected in our system are indeed those utilised during normal chromosomal DNA replication.

Our work allows further isolation and subsequent sequence analysis of any desired number of yeast chromosomal replication origins. It is hoped that information about the nature of replication origins will aid studies of the biochemical mechanism of initiation of eukaryote DNA synthesis.

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Is 3'-nucleotide rigid?

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Conformational analysis of nucleic acids and polynucleotides is far more complex than that of proteins and polypeptides, due to five single bond rotations in addition to the sugar puckerings in the monomer. Sundaralingam¹ proposed the concept of the 'rigid' nucleotides from analysis of crystal structure data, with the flexibility allowed only about the phosphodiester bonds. However, the crystal structure of deoxyguanosine-5'-phosphate^{2,3} indicates at *gt* conformation about the C-4'-C-5' bond against *gg* in a conformationally rigid nucleotide⁴. Jack *et al.*⁴ considered the flexibility of nucleotides in tRNA about the C-4'-C-5' bond, thereby introducing the concept of 'non-rigid' ribonucleotides. Conformational flexibility of the furanose ring in DNA and RNA and their energetics using classical and quantum chemical methods have been reported^{5–8}. We have examined the flexibility of 3'-nucleotides. α , the most important of the conformational parameters defining the 3'-end of a nucleotide unit⁹, has a value in the range 195°–270° in all the 3'-nucleotides, dinucleoside monophosphates and higher oligomers which have been surveyed. A survey of the proposed structures of polyribonucleotides^{10,11} also shows the values of α to be greater than 200°. However, the structures proposed for B-DNA by Arnott and Hukins^{12,13} and D-DNA by Arnott *et al.*¹⁴ have values of α of 155° and 141° respectively, much lower than the lowest observed value. The structure for B-DNA has two strong, short contacts (C-2'...OP-1 = 2.64 Å and HC-2'...OP-1 = 1.79 Å) which lead to an energetically unfavourable conformation. Hence, it is of interest to investigate whether, by allowing flexibility to the sugar moiety in the nucleotide unit, it is possible to make the structure energetically favourable. Here, conformational energy calculations were carried out to determine the range of α which would give rise to energetically favoured conformations with different sugar puckerings. Our analysis has shown that the theoretically obtained range is nearly the same as the preferred range in crystals, indicating the flexibility of the 3'-nucleotides.

Conformational energy calculations were carried out using classical potential functions¹⁵ for the sugar-phosphate fragment (Fig. 1a) of the nucleotide unit for both ribose and deoxyribose sugars. The sugar geometries were chosen from the minimum energy regions of the pseudo-rotation space^{7,8}. For each of the

values of Φ (defined in Fig. 1b legend), energy was minimised for each value α over a few geometries. Φ was varied from 0° onwards at intervals of 18°, and α from 120° to 300° at intervals of 10° and 1° (in the minimum energy region). Outside the specified range for α , for all geometries considered, strong short contacts were observed between the atoms in the phosphate group and the groups of atoms attached at the C-5' and C-2' atoms. The torsion angles β and ϵ were fixed respectively at their average values of 290° and 50° (ref. 16). The other two pendant oxygens were fixed tetrahedrally. Energies were also obtained for *gt* and *tg* conformations about the C-4'-C-5' bond. The general features of the Φ - α plot do not change for small changes from the average values of β and ϵ . However, for the *tg* conformation, it was observed that the C-3' *endo* region does not have energetically favourable conformations for both deoxyribose- and ribose-phosphate fragments. Details of this will be published elsewhere.

Figure 2 shows the energy contours at an interval of 1 kcal mol⁻¹ from the minimum in the Φ - α space for the ribose-phosphate fragment. Observed values for α in many crystal structures are also plotted. The plot shows the global minimum

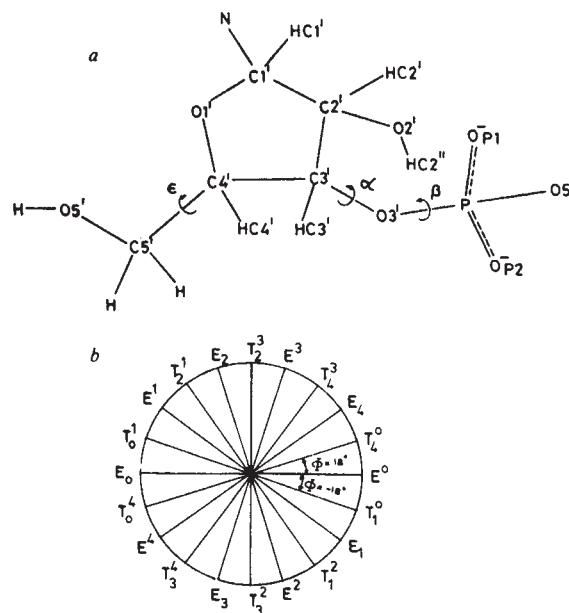


Fig. 1 a, The ribose-phosphate fragment of a ribonucleotide. b, Definition of the pseudo-rotation phase parameter in the pseudo-rotation space.

at $\alpha = 280^\circ$ in the C-2' *endo* region. The minimum has been indicated by a vertical line, as, along this line energy does not vary significantly. The energy varies differently in two directions about the minimum. On the left ($\alpha < 280^\circ$), the energy rises very gradually as α decreases. On the right ($\alpha > 280^\circ$), as α increases, the energy increases very rapidly due to strong unfavourable non-bonded interactions between atoms in the phosphate group and hydroxyl group and hydrogen atoms at C-5'. Apart from the global minimum at $\alpha = 280^\circ$ in the C-2' *endo* region, the plot also shows two local minima in the C-3' *endo* region at $\alpha \approx 200^\circ$ and $\alpha \approx 270^\circ$. Note also that transitions are possible from the C-3' *endo* to C-2' *endo* regions via the O-1' *exo* region at high values of α by crossing an energy barrier of 1 kcal mol⁻¹.

Figure 3 shows a similar plot for the deoxyribose-phosphate fragment. As in ribose, there is a global minimum in the C-2' *endo* region at $\alpha = 280^\circ$ (indicated by a vertical line for reasons stated above). Also as in ribose and for reasons already stated, this plot indicates a rapid rise of energy as α is increased to the right of the global minimum. To the left ($\alpha < 280^\circ$) there is a gradual rise in the energy. However, unlike in ribose, there is another local minimum around $\alpha \approx 190^\circ$ in the C-2' *endo* region in addition to two local minima in the C-3' *endo* region (at $\alpha \approx 180^\circ$ and $\approx 270^\circ$). The plot also shows that conformational transitions between the C-3' *endo* and C-2' *endo* regions are possible via both O-1' *exo* and O-1' *endo* regions. At low values of α ($\approx 200^\circ$) the barrier to transition via either O-1' *exo* or O-1' *endo* is 2 kcal mol⁻¹. At high values of α ($\approx 270^\circ$) the barrier via O-1' *exo* is 1 kcal mol⁻¹ and is very high via O-1' *endo*.

A comparison of the Φ - α plots for ribose- and deoxyribose-phosphate fragments shows a larger conformational flexibility in the case of the latter. At low α values ($\approx 180^\circ$), C-2' *endo* region is prohibited for ribose because of strong short contacts between the bulky OH group at C-2' of the sugar and the atoms in the phosphate group. These are relieved to some extent in deoxyribose, where only hydrogen is attached at C-2'. This explains the occurrence of a local minimum at $\alpha \approx 190^\circ$ in the C-2' *endo* region. However, for both the sugars, we noted that in both the C-3' *endo* and C-3' *exo* regions, very low values of α , such as 140° - 150° , are prohibited. Energy contours as high as 4-5 kcal mol⁻¹ pass through the regions at such low α values. In the C-3' *endo* region the local minimum at $\alpha \approx 200^\circ$ in the case of ribose shifts by about 20° on the lower side in the case of deoxyribose; this is due to the presence of hydrogen at C-2' instead of a bulky hydroxyl group. The comparison also reveals that transitions between C-3' *endo* and C-2' *endo* regions are very restricted in ribose, but are relatively easy in deoxyribose. Whereas in ribose only high α transitions are possible via the O-1' *exo* region, both low and high transitions via the O-1' *exo* and O-1' *endo* regions are possible for deoxyribose.

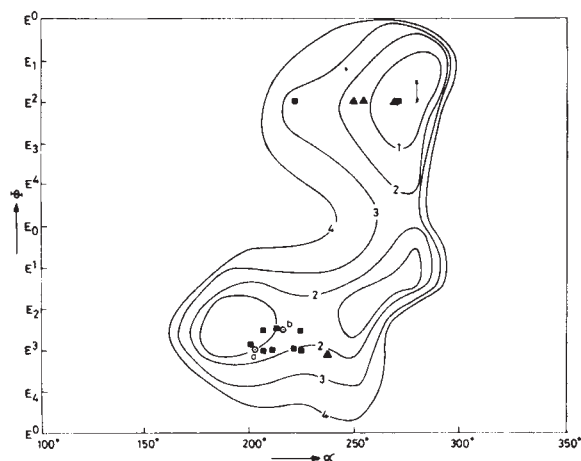


Fig. 2 Φ - α plot for the ribose-phosphate fragment. Energy contours of 1.5 kcal mol⁻¹ in the C-3' *endo* region are shown. ▲ Denotes 3'-nucleotides, ■ denotes dinucleoside monophosphates and higher oligomers, ○ denotes proposed structures of polynucleotides. ○^b, RNA-11 (ref. 11); ○^a, A-RNA (ref. 10).

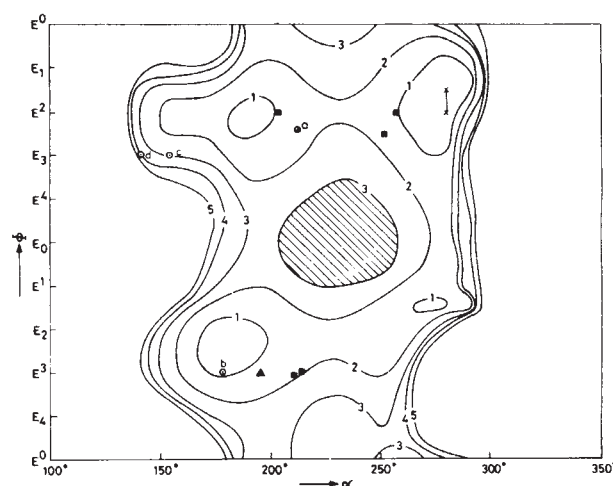


Fig. 3 Φ - α plot for the deoxyribose-phosphate fragment. ▲ Denotes 3'-nucleotides, ■ denotes dinucleoside monophosphates and higher oligomers, ○ denotes proposed structures for polynucleotides. ○^a, C-DNA¹⁸; ○^b, A-DNA¹⁷; ○^c, B-DNA^{12,13}; ○^d, D-DNA¹⁴.

It is found that for compounds containing ribose sugars, values of α mostly range between 200° and 240° in the C-3' *endo* region and between 220° and 270° in the C-2' *endo* region. Two of the proposed structures for polynucleotides (A-RNA and RNA-11)^{10,11} also lie in the C-3' *endo* region. In the case of deoxyribose sugar compounds, the observed points are spread over a wider range of α than in ribose in both C-3' *endo* and C-2' *endo* regions. It is found from the plots that energetically favourable conformations are possible with $\alpha > 180^\circ$, and that for structures with $\alpha < 150^\circ$ unfavourable conformations result. The refined model of B-DNA¹³ ($\alpha = 155^\circ$) lies on the 3 kcal mol⁻¹ contour in the C-3' *exo* region of the Φ - α plot for the deoxyribose-phosphate fragment. However, β and ϵ in the refined structure of B-DNA are different from those used in the present calculations. Conformational energy calculations carried out using the conformational parameters of the refined model reveal that the model is 4 kcal mol⁻¹ above the minimum and thus energetically unfavourable. Further, V.S. has pointed out that the structure is 2 kcal mol⁻¹ above the minimum in the Φ - χ plot⁷, which emphasised the influence of the sugar geometry on the value of χ , the glycosidic torsion angle. Considering the nucleotide unit as a whole, the refined model of B-DNA¹³ is more than 5 kcal mol⁻¹ above the minimum despite allowance for the flexibility of the sugar unit.

We have used these results to produce a refined model for B-DNA which differs from that of Arnott and Hukins¹³. We have refined a right-handed double-helical model which is in good agreement with the X-ray diffraction data and has an energetically favourable conformation. We also find that the left-handed double-helical model for B-DNA is energetically favourable and is in good agreement with the X-ray diffraction data. The details of the two structures are published elsewhere.

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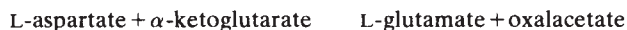
Electron density map of chicken heart cytosol aspartate transaminase at 3.5 Å resolution

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Aspartate transaminase (EC 2.6.1.1., Asp-transaminase) has been studied extensively, and much is now known about its physico-chemical, catalytic and other properties¹. X-ray studies that can provide a structural foundation for the events that occur during the transamination reaction are under way on three species of Asp-transaminase: the cytosolic enzyme from pig² and chicken³ hearts, and the mitochondrial chicken heart enzyme⁴. We describe here the interpretation of an electron density map of Asp-transaminase from chicken heart cytosol at 3.5 Å.

Aspartate transaminase catalyses the reaction



The enzyme is dimeric (molecular weight 93,000), consisting of two chemically identical subunits. Crystals were grown in the presence of α -methyl aspartate (α -Me-Asp) as previously described³. Their space group is $P2_12_12$, with cell dimensions $a = 62.7$ Å, $b = 118.1$ Å and $c = 124.5$ Å, and they contain one dimer in the asymmetric unit⁵. The 3.5 Å resolution map was calculated using phases obtained from three isomorphous derivatives, improved by making use of the non-crystallographic symmetry relating the two subunits in the molecule⁶. Other states of the enzyme were obtained by soaking experiments, and examined at lower resolution using difference maps.

Tracing the polypeptide chain was facilitated by the extensive secondary structure present in each subunit. Nine α -helices were located, including one 48 Å long, and a number of β -strands, five of which form a parallel β -sheet in the core of the subunit. Figure 1 shows part of the electron density map including the long helix and part of core β -sheet. It was possible to interpret the electron density map in terms of a unique chain tracing, a schematic drawing of which is shown in Fig. 2. According to the current classification of globular proteins⁷, Asp-transaminase is an α/β protein. Figure 2 shows that the subunit is composed of three parts of about equal size, defined by the preferential directions of the α -helices and β -strands. The three directions are almost mutually perpendicular. Of these three parts, only one, shown in the lower centre of Fig. 2, can be considered as a separate domain, the others are combinations of fragments from the N and C termini. The separate domain shows a close resemblance to the well-known 'nucleotide binding domain', first discovered in the NAD-dependent dehydrogenases^{8–11}, and then found in a number of other enzymes including phosphoglycerate kinase¹², phosphoglycerate mutase¹³, rhodanese¹⁴ and glycogen phosphorylase^{15,16}. In Asp-transaminase this domain is composed of five parallel β -strands alternating with five antiparallel helices. Figure 3 shows a drawing of this domain in comparison with the NAD-binding domain of lactate dehydrogenase^{17,18}. Interestingly, both pyridoxal phosphate-containing enzymes studied by X-ray analysis (Asp-transaminase and glycogen phosphorylase) have such a domain in their structure; other pyridoxal enzymes may also contain domains of this kind.

The enzyme's active site was first located from a difference map of the apoenzyme at 5 Å resolution³. At 3.5 Å resolution it can be concluded that the negative density seen in the difference map between the native and apoenzyme corresponds only to the pyridine moiety of the coenzyme; the phosphate moiety is probably replaced by a dianion from the solvent in the apoenzyme crystals. The phosphate group of the coenzyme has been recognised in the 3.5 Å native map as the highest density peak located close to the previously identified pyridine ring. The coenzyme is located at the carboxy end of the β -sheet of the nucleotide-binding unit, with its phosphate group at the N terminus of the first upper helix as shown in Fig. 2, suggesting an interaction of the charged phosphate with the helix dipole¹⁴. The coenzyme is located about 7 Å from the subunit interface, represented by the upper face of the drawing in Fig. 2. The substrate binding region, deduced from a series of difference maps (see below) is placed between the coenzyme and chain segments from the N and C termini (see Fig. 2). Overall the active site is located at the interface of the three parts of the subunit noted above.

Spectroscopic studies on crystals of pig cytosolic enzyme¹⁹ suggested that the complex with α -Me-Asp is a mixture of two spectroscopically discernable forms, probably representing differences in the coenzyme orientation. However, in the X-ray studies the electron density for α -Me-Asp can be accounted for by a single conformation and a fixed geometry of the substrate analogue in both forms can be assumed. The density of the pyridine ring is slightly less pronounced but the span of possible orientations is only 15–20°. We have shown previously³ that these crystals of Asp-transaminase are catalytically competent, allowing us to examine different states of the enzyme. Previous studies³ have shown that crystals washed free of α -Me-Asp, and colourless crystals of the pyridoxamine form of the enzyme gave 5 Å resolution difference maps containing similar features interpreted as conformational changes in the enzyme. Now it can be seen that these changes are located in a wide region including the substrate-binding area and the C-terminal helix. It seemed possible that the conformational pattern in this area is determined by the presence of substrate carboxyl groups. To check this the complexes with erythro- β -hydroxyaspartate, maleate and succinate have been studied.

Crystals were soaked in a stabilising solution³ (without α -Me-Asp) with the addition of one of the three mentioned

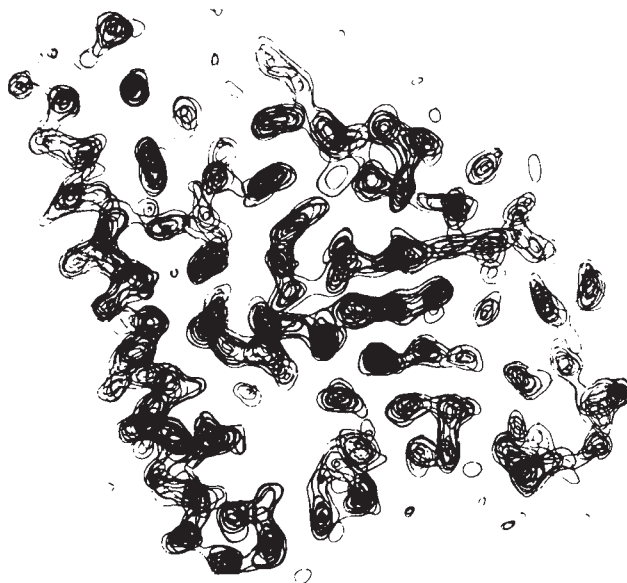


Fig. 1 Superposition of the eight sections of the electron density map of Asp-transaminase from chicken heart cytosol (at 3.5 Å resolution). The sections are perpendicular to the z axis, with the distance of 0.8 Å between neighbouring sections. A big helix 48 Å long is seen at left side. The horizontal β -strands of a parallel β -sheet in the centre are a part of the 'nucleotide-binding domain'.

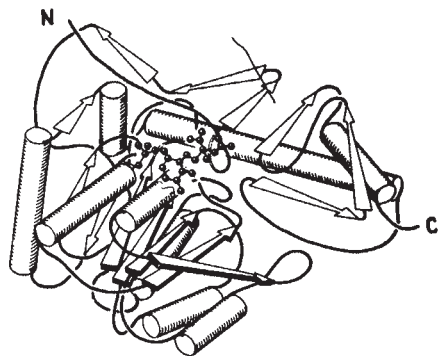


Fig. 2 Schematic drawing of the model of Asp-transaminase subunit built from secondary structure elements. α -Helices are shown as cylinders and β -strands as arrows, those belonging to the 'nucleotide-binding domain' shown as space-filled arrows. The coenzyme is shown covalently linked to α -Me-Asp.

compounds, at 50 mM concentration. Crystals soaked with erythro- β -hydroxyaspartate acquired a gingerish colour after 1 hour, which could be maintained for several months. The X-ray experiments were performed on crystals soaked for 2 h. Crystals soaked with maleate or succinate became a distinctly brighter yellow than the original α -Me-Asp complex. X-ray experiments were done after 1–2 days of soaking. At 5 Å resolution, the difference maps of all three complexes contained some features in the substrate-binding area but no conformational changes. A similar X-ray experiment on the abortive complex of the pyridoxal form of the enzyme with 2-oxoglutarate gave a difference map showing conformational changes localised in the same region as the substrate-free form of the holoenzyme³, but of opposite sign.

It is of interest that the conformations of the apoenzyme and the α -Me-Asp complex are similar. A number of prominent positive peaks observed near more extended features interpreted as conformational changes, suggest that some of the conformational changes can be ascribed to the interaction of ions. After the removal of α -Me-Asp one such positive peak persists near a position previously occupied by the distal carboxyl. Another is seen in the vicinity of the C-terminal helix. It can be supposed that an anion replacing the substrate carboxyls is a cause of the conformational changes in the substrate-binding area. Also, in relation to the apoenzyme, the absence of negative density for the removed phosphate of the pyridoxal phosphate or substrate analogue may be explained by similar anion replacements. It is possible that the additional space created by removal of the coenzyme allows the anion to bind without causing conformational distortions to the adjacent protein framework.

In the study of Asp-transaminase much attention has been paid to the so-called syncatalytic phenomena observed after the addition of pairs of substrates (such as the L-glutamate/2-

oxoglutarate pair)^{20–24}. These effects have been treated in terms of conformational changes concomitant with the formation of covalent intermediates in the course of the catalytic process. It is tempting to assume that the conformational changes discussed here are a part of the same picture. However some remarks seem to be relevant: (1) The conformational changes observed by us are concomitant not with catalysis but with binding of substrates or inhibitors. (2) The conformational changes that accompany the transition between substrate-binding, and substrate-free forms are different in two subunits, this asymmetry may be explained by restrictions imposed by crystal packing. (3) The possibility that the observed changes are a property only of the chicken heart cytosol enzyme cannot be excluded. (4) In addition to the gross conformational changes observed at 5 Å resolution, more subtle changes are probably also present which may be of the most important functional meaning. It may be predicted that comparative X-ray studies of different states of Asp-transaminase at higher resolution should soon clarify many of the problems discussed here.

We thank Yu. V. Nekrasov, A. A. Vagin and V. M. Kochkina for valuable assistance, and L. N. Johnson for the picture of 'nucleotide-binding domain' in phosphorylase *b*. We also thank Professor A. E. Braunstein for valuable advice and discussions. *Note added in proof:* Recently similar three-dimensional structure and conformational changes have been described for chicken mitochondrial Asp-transaminase²⁵.

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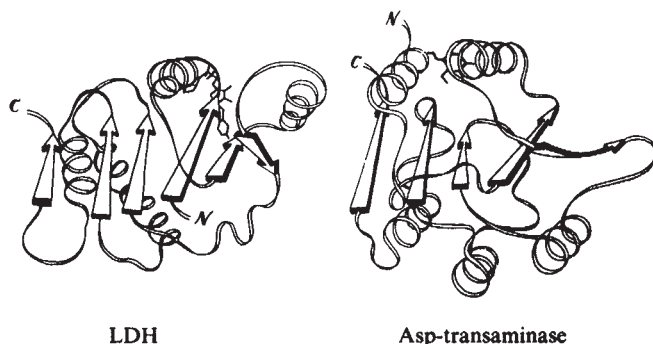
Corrigendum

In the News and Views item 'Structure and force generation in muscle' by J. M. Squire, *Nature* **281**, 99, lines 14 and 15 in the right-hand column should read '... of 20% of the actin monomers in the thin filaments or to 40% labelling...' (The values 20 and 40% were transposed in the original.)

Erratum

In the review article 'Nuclear weapons and power-reactor plutonium', by A. B. Lovins, *Nature* **283**, 817–822, p. 818 paragraph 1 line 4, should begin '(GWt-d)', paragraph 2 line 6 should read '... 27–33 GWt-d/T...'. A line was omitted from the paragraph on p. 822 headed Conclusions. Lines 10–17 should read:

'and "not a valid concept"³¹. (Dilution with UO₂ or other materials requiring chemical or physical⁵⁶ separation is a valid concept—it means more material must be diverted and processed^{19,25,34}—but does not solve the problem.) Taking all effects on weapons physics into account, a high ^{238,240,242}Pu content may reduce expected yield to a level that could devastate only a modest portion of a city rather than all of it, and may make that yield much less predictable, if the bomb is crudely made. But...'. In refs 27 and 55 the title should read 'Nuclear Policies: Fuel Without the Bomb'.



LDH

Asp-transaminase

Fig. 3 Comparison of two domains present in the three-dimensional structure of lactate dehydrogenase and Asp-transaminase.

MATTERS ARISING

Information transmission in remote viewing experiments

IN a recent letter to *Nature*¹ Marks and Kammann offer criticism of the SRI experiments in 'remote viewing', the ability of certain individuals to access and describe, by means of mental processes, information blocked from ordinary perception by distance or shielding²⁻⁴. They hypothesise that the apparent success in these experiments may simply be an artefact of statements in the subjects' transcripts which provide extraneous cues useful to judges attempting to blind match transcripts to target sites. They then argue that examples from the transcripts of the first published experiment—a nine-trial series with subject Price²—support their hypothesis. We present here experimental evidence that demonstrates that this conjecture is false.

On reading Marks and Kammann, one of us (C.T.T.), who had no connection with the original Price series, offered to independently reanalyse that series to test the validity of the Marks–Kammann hypothesis. He edited the transcripts carefully, removing all phrases suggested as potential cues by Marks and Kammann, and removing any additional phrases for which even the most remote *post hoc* cue argument could be made. The series was then rejudged by a new independent qualified judge (having previously shown competence in blind matching and verbal content analysis of similar materials) who was unfamiliar with the Price series. The materials turned over to the judge consisted of the newly edited transcripts presented in random order, and the list of target sites, also in random order (different from both the transcript random order and from the order of original target usage). The judge was instructed to provide, on a blind basis, a set of target/transcript correspondence ratings, which required that she visit each target site and rate transcripts to targets on a scale of 0–100 for all possible combinations, generating a 9×9 matrix. These data also yield the conventional overall measure of target/transcript correlations by indicating each first place match from the set of nine for each target.

The result of the blind matching of transcripts to target sites in the rejudging was that seven of the nine were again correctly matched. The appropriate statistic for this overall matching result is derived assuming non-independent assignment of transcripts to target sites (as in guessing the order of a random sequence of the digits one through nine, each used once)⁵; the result (seven out of nine correctly matched) is significant at

$P < 10^{-4}$. Furthermore, the more detailed target/transcript rating matrix was analysed by an exact factorial method⁶; the result obtained by this analysis was significant at $P = 2.2 \times 10^{-5}$. The greater significance obtained by the latter, more sensitive measure of target/transcript correlations reflected the following important fact: the judge found that, with the exception of two transcripts that did not seem to correspond to any site, the remaining seven transcripts each showed high correlation to one (correct) site and low correlation to the others. This is in direct contradiction to the implication of Marks and Kammann that little target/transcript correlation existed, and that matching is possible only on the basis of artefactual cues.

Therefore, on the basis of an independently conducted empirical test, we reject as invalid the Marks–Kammann conjecture that success in the first-published study on remote viewing is to be attributed to cueing artefacts rather than to transcript/target correlations. It is also important to note that the Marks–Kammann critique did not address the quality of the remote-viewing descriptions in the transcripts *per se*, but was instead limited to criticism of a particular judging procedure used to evaluate those descriptions. With regard to the descriptions themselves, we note that in the nine-transcript series in question, when the target was a boat marina the subject gave a consistent narrative that began with "What I'm looking at is a little boat jetty or boat dock along the bay. It is in a direction about like that (pointing) from here. Yeah, I see the little boats, some motor launch (sic), some little sailing ships. . . ." For a landmark Hoover Tower site, the subject summarised his impressions as "The area—I have a place—seems like it would be Hoover Tower". For a recreational swimming pool site with a 75 ft $\times$ 100 ft rectangular pool and a 110 ft diameter circular pool, the subject made a drawing of the target area as centred about two pools of water, which he dimensioned as a 60 ft $\times$ 89 ft rectangular pool and a 120 ft diameter circular pool; and so forth. Furthermore, as pointed out above, blind content analysis of the transcripts, which provides a sensitive measure of the degree of target/transcript correspondences, confirms objectively the subjective impression of above-chance correspondence that one infers from examples such as the above. With data of this quality we would argue that it is not surprising that empirical test failed to confirm the cueing-artefact hypothesis put forward by Marks and Kammann, but rather confirmed that the target/transcript matches in the first remote-viewing

study are to be attributed (as originally interpreted) to the quality of the subject's descriptions themselves.

Furthermore, in the extensive SRI replication studies³, which also yielded significant results, the Marks–Kammann criticisms do not apply in principle. Target lists and transcripts were separately randomised, and transcripts were carefully checked before judging to ensure absence of any phrasing for which even a weak *post hoc* potential-cue argument could be made.

Given (1) the failure (by empirical test) of the cueing-artefact hypothesis to account for the success of the first-published SRI remote-viewing study², (2) the inapplicability of this hypothesis to the later SRI replication studies³, and (3) the replication of this work in our own and other laboratories, the bulk of which is successful⁷, the data continue to confirm the original conclusion that remote viewing is a viable human perceptual capability.

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Is Cassiopeia A a black hole?

SHKLOVSKY has recently¹ summarised evidence in favour of a black hole in Cas A in contrast to my arguments^{2,3} in favour of a binary neutron star. I defend my view here.

The fact that Cas A has not been reported as a supernova is disturbing as it has an assumed interstellar absorption A_v of 4.3 mag, and a distance of 2.8 kpc. On the other hand, Tammann⁴ has suggested a mean supernova rate of some $10^{-1.1 \pm 0.3} \text{ yr}^{-1}$ for our Galaxy; a similarly high rate is needed to account for the observed neutron stars, both pulsars and transient X-ray sources. These estimates may be reconciled if supernovae are sometimes obscured, by an adjacent dense hydrogen cloud, say, whose absorptivity is

temporarily raised by ionisation. Note that such an explanation would similarly be able to explain the inclination dependence⁴ of supernova rates in external galaxies, and the absence so far of Type 2 explosions in irregular galaxies.

Cas A is anomalously bright at radio frequencies, and one is tempted to conclude that it has an anomalous mass. From optical observations, one infers a mass $<1 M_{\odot}$. If this mass is concentrated in a network of filaments, of electron density $>10^3 \text{ cm}^{-3}$, then the X-ray luminosity can similarly stem from a mass $<1 M_{\odot}$. (McKee's assumption of homogeneous shells need³ not be realistic.) And if the velocity field of the quasi-stationary flocculi reveals³ a peculiar velocity of $(165 \pm 15) \text{ km s}^{-1}$ of the whole remnant, towards north-northeast and towards us, then the supernova remnant must be lighter than its recoil partner (unless the latter moved even faster), again supporting a mass $\leq 1 M_{\odot}$ in the conservative case.

This recoil partner cannot be very luminous because it has not even been detected. Very careful searches have been performed near the divergence centre of the optical knots, whereas the recoil may have removed it southwards. I think that a low mass binary containing a neutron star is a likely candidate. The neutron star's surface temperature⁵ may be anything between 10^5 K and 10^7 K , depending on pion condensation in its interior. Its relativistic wind may be shielded by a cocoon formed from the wind material of its companion. And it can be the source of the cosmic rays in the nebula, and of the soft radio flare⁶ which has probably occurred between 1967 and 1974. Alternatively, the recoil partner may be a second binary pulsar⁷.

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Evidence for late Precambrian plate tectonics in West Africa

BLACK ET AL.¹ have reported further evidence for plate tectonics in the Precambrian based on their study of a 600-Myr-old collision zone proposed^{2,3} along the eastern margin of the West African craton. In places, this margin is associated with a negative-positive gravity anomaly pair⁴. A case for plate tectonics in the Canadian Shield, following the initial suggestion⁵ and a description of the geological evidence⁶, has centred around interpretation of similar anomalies

present at several structural boundaries proposed as collisional suture zones⁷⁻¹⁰. The type structure derived from the anomalies comprises an older crustal block, that thickens towards the suture, in steep contact with a thicker and denser, younger crustal block, both blocks being in approximate isostatic equilibrium¹¹. A similar model has been proposed^{12,13} for the eastern margin of the West African craton. These gravity models (Canadian and West African) are comparable with a 'basement reactivation' model proposed for collided continents¹⁴.

We include the West African anomalies in a growing family of anomalies that seem to be collision-related phenomena reflecting a particular crustal configuration developed at suture zones. The gravity anomalies in the Canadian Shield occur along 1,800 and 1,000 Myr old orogenic belts. The addition of a 600 Myr old example from Africa suggests a continuum throughout most of Proterozoic time (1,800–600 Myr BP). Another example of paired anomalies, currently under study, along the southern Appalachians apparently extends the continuum into the Phanerozoic to ~450 Myr BP. Similar gravity signatures have been described from the Precambrian shields of Australia¹⁵ and India¹⁶. Precambrian collisions proposed in Europe and South America¹⁷ may provide more examples.

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BLACK ET AL. REPLY—We agree with Thomas et al. on the importance and significance of paired gravity anomalies

along collisional suture zones, although we feel that detailed seismic information is required to perfect the model.

Note, however, that the anomalies along the eastern edge of the West African craton fall into two categories: (1) anomalies of long wavelength for which one can envisage a crustal structure similar to that proposed by Thomas et al. to explain coupled negative and positive anomalies along a cryptic suture. In Togo–Benin, however, in assessing the negative anomaly, one has to take into account the sediments of the passive continental margin (Buen, Atacorian). (2) Positive anomalies of shorter wavelength corresponding to more superficial unrooted structures and displaying subvertical to easterly dipping geometry in accord with the general movement pattern with thrusting towards the craton. Field observations show that these anomalies are due to basic and ultrabasic bodies emplaced along the suture^{1,2}.

The location of the two types of anomalies seems to be related to the geometry of the craton, which we believe reflects the original shape of the continent before collision. The first type of paired gravity anomalies occurs in Togo–Benin where the craton forms a promontory and where collision is thought to have been most intense. This situation led to the complete disappearance by subduction of the oceanic floor and of the active margin to the eastern continent, thus bringing into direct contact by underthrusting the low-grade metasediments belonging to the passive continental margin of the craton, and high-grade basement of the eastern continent. In contrast, the second type of anomalies are located in an embayment where island arc and cordilleran volcanoclastic assemblages of the active margin to the eastern continent have been preserved.

The Bouguer anomaly map of Canada suggests that several paired gravity anomalies are arcuate in form and occur along promontories, such as the Thelon front between the Slave and Churchill provinces³.

We believe at present that the stress and deformation pattern together with the gravity signature have largely been controlled by the geometry of the colliding passive continental margin^{4,5}.

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BOOK REVIEWS

Essay-writing is difficult

Eric Ashby

"THE essayist," wrote E. B. White, himself a virtuoso in essay-writing, is "sustained by the childish belief that everything he thinks about, everything that happens to him, is of general interest." Essays can be classified as those written to instruct, to persuade, or to entertain. If they have to be limited in length to some 1,200 words, preferably about some scientific topic, they are a difficult art-form for any of these three purposes. This is what Professor Morowitz attempts in this book. It is an assemblage of 57 titbits from the magazine *Hospital Practice*: some to instruct, some to persuade, and all — the author himself confesses — intended to entertain.

To be fair to these essays it is essential to remember that they appeared one at a time, as appetisers, in a magazine devoted (to judge from its title) to the sober topic of how to care for the sick. To offer the reader 57 appetisers, all at once, undiluted by articles on pre-natal clinics and intensive care units, is to expose the appetisers to unreasonable criticism. Only a master in essay-writing could hope to stand up to such criticism. The blurb on the dust-cover "invites comparison to *Lives of a Cell* by Lewis Thomas." This is not fair, for pleasant as some of Morowitz's whimsical pieces are, he is not in the same league as Lewis Thomas, for reasons I shall try to summarise at the end of this review.

Here is a sample of the more enjoyable essays. Morowitz receives a birthday card with the caption: "According to biochemists the materials that make up the human body are only worth 97 cents." He goes through the catalogue of a biochemical company and finds that the market price of the compounds in his body, synthesised from the 97 cents-worth of elements, is of the order of 6 million dollars: a nice reminder that "information is much more expensive than matter." The bicentennial of the United States in 1976 moves him to reflect that 1976 is also the centennial of "the second most significant document produced in the United States, Josiah Willard Gibbs' paper *On the Equilibrium of Heterogeneous Substances*: and there follows an epitome of Gibbs' contribution to science. In another essay he describes a conference of

The Wine of Life, and Other Essays on Societies, Energy and Living Things. By H. J. Morowitz. Pp.265. (St Martin's Press: New York, 1979.) \$10.

ecologists at the Hague and is shocked at the way they fouled the air with cigars distributed at a reception in their honour. This was an unfortunate theme to choose, for it does invite comparison with Thomas' essay on a medical conference at Atlantic City (*On Societies as Organisms*). Lewis flutters like a graceful bird over the scene of the conference; Morowitz (it seems to me) wades through the scene in gumboots. The essayist (if he has the skill) can be Mozartian in 1,200 words; he cannot, in 1,200 words, be Wagnerian.

Occasionally Morowitz does bring it off. He picks on a phrase in a report about experiments on animals: "the animals were sacrificed." This, in Britain, could mean simply that the worker was complying with the requirements of the Cruelty to Animals Act of 1876. But think (writes Morowitz) about the meaning of the word 'sacrifice': "the slaughter of an animal as an offering to God or a deity." He draws a parallel between the practice of animal sacrifice in religions, which is being phased out, and animal sacrifice in the cause of science, which is on the increase: the incinerator replacing the burnt offering, the control group of rats replacing the Old Testament goat which was not slaughtered; the purpose, however, remaining the same: to "ward off disease, illness, and old age . . .". This is excellent satire, and timely for British readers, for there are at present two Bills before Parliament for the reform of the Cruelty to Animals Act of 1876.

The purpose of some of the essays is to debunk, and Morowitz does this well, if a little heavy-handedly. He publishes a nice letter he wrote to his hairdresser, who tried to persuade him to have his hair treated with a conditioner containing nucleic acid (at, presumably, exorbitant cost). So he considers just what effect applications of DNA or RNA might have on the scalp, and he ends by advising his hairdresser to beware of being an agent for the abuse of words like nucleic acid, proteins, lecithins,

and other mumbo-jumbo borrowed from science to promote profits in the cosmetic industry.

One more example: the essay from which Morowitz chose the title for his book. It is as neat an account as one could get into four pages of the way Claude Bernard (whose home was in the Beaujolais region of France) and, after him, Pasteur, were inspired to do some of their best work by considering "how grape juice becomes wine". This is the use of the essay to instruct and it is as instructor that Morowitz is at his best. He is undoubtedly well read, scholarly, and — consistent with E. B. White's prescription — he wants to tell you what has interested him since the last issue of *Hospital Practice*.

Why, then, do I have reservations (not shared by reviewers quoted on the dust-cover) about the quality of these essays? I have two reasons. One is that essays, unlike articles, published lectures, and monographs, have to be judged primarily upon felicity of style. A good essay sparkles with surprises; it captures your attention without being importunate; it can (Dr Johnson said this about essays) be "an irregular, undigested piece" but it has to convey delight, like a kitten playing with a ball of wool. To do this at all is difficult; to do it on a scientific theme is extremely difficult. I don't find Morowitz's style felicitous: a subjective judgement, of course, and maybe it's my fault and not his.

Second, the essay, unlike other kinds of writing, should tell you a lot about the essayist. After reading Montaigne, Lamb, Jorge Borges — and in science T. H. Huxley, J. B. S. Haldane, and Lewis Thomas — you feel you know the essayist: his prejudices, his enthusiasms, what he stands for. The relation between reader and writer has some of the qualities of friendship. Irregular and undigested as essays may be, they add up to the portrait of a distinct personality. This is what I missed in *The Wine of Life*. □

Lord Ashby is Chancellor of Queen's University, Belfast, and a Fellow of Clare College, Cambridge, UK.

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British stratigraphy

F. Wolverson Cope

A Dynamic Stratigraphy of the British Isles. By R. Anderson, P.H. Bridges, M.R. Leeder and B.W. Sellwood. Pp.301. (George Allen and Unwin: Hemel Hempstead, UK, 1979.) Hardback £15; paperback £7.95.

APTLY titled, this book describes the evolution, over a period of about 3,000 million years, of that part of the Earth's crust occupied by the British Isles.

The past two decades have seen a revolution in geological thinking. Major factors in this have been the increasing capability to date rocks radiometrically, the study of palaeomagnetism, remarkable advances in sedimentological interpretation based upon increased knowledge of modern sedimentary processes, the exploration of sea and ocean beds and of the crust beneath them, and the theory of plate tectonics which has arisen from these. This is an appropriate time for some stocktaking.

The authors are to be congratulated on having so successfully re-interpreted, indeed in some cases interpreted for the first time in a meaningful way, the many phases in the history of the British Isles area. They have explained the evolution of this part of the Earth's crust in the light of plate tectonics, and have added considerably to the story by using results of hydrocarbon exploration in the North Sea.

With a necessarily chronological

treatment, the reader will gain a broad appreciation of the stratigraphy of the British Isles and the surrounding seas. The book highlights the value of sedimentological studies in stratigraphy. Fossils are used almost exclusively as ecological indicators; the results of their use in correlation are taken very much as read so that some stratigraphical problems are glossed over.

The account is well written. It is profusely illustrated with carefully executed maps and diagrams, and the volume is pleasant and easy to use. Though not advocating expansion in technical vocabulary, one feels that a tautological expression such as "facing confrontation" (page 165) should be eliminated. On the other hand, technical terms which come so readily to hand are sometimes better avoided as in "Perhaps palaeoslopes were directed southwards in this area" (page 234) when speaking of slopes.

This book should be invaluable to students and others who wish to gain some understanding of the evolution of the Earth's crust in the British Isles area. It should serve many generations as a useful framework of spatial and dynamic relationships without which stratigraphy must fail to inspire.

Adopting a metaphor suggested by non-use of the adjective *seminarial* (in the gloss on the back of the book), one would say that the four authors have combined most successfully in the production of a lively 'zygote', especially as it might never have come about but for "... a seminal [*sic*] encounter"! □

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Chinese revelation

M. Christine King

China's Road to Development. Edited by Neville Maxwell. Pp.365. (Pergamon International Library: Oxford, 1979.) Hardback £21; paperback £5.25.

It is a strange fact of life that those most responsible for making history have always been the least able to record it for posterity. The result of this is that, from time to time, history has to be re-written. The present volume on China, an enlarged edition of a collection of essays first published in 1976, is perhaps an apposite example of this phenomenon. It appears at a time when China is deliberately revealing its own past in order to point a way to the future. Thus many of the 'development' theories outlined in the work already form part of history, a past which must now be re-examined. Few are more aware of this than the editor, who has the courage to admit in

his prefatory note that "the papers in this collection have to some extent been outdated".

The book consists of 16 essays by different authors covering developments in China since 1949 as seen in its entirety from the western point of view. Between them the authors cover rural development, industrialisation, agriculture, health care and population control, economic planning and trade, environmental protection, women's role in the country's development, forestry, intermediate energy technology, Chinese foreign aid, city planning and the role of the People's Liberation Army. Curiously, the place of science, the very foundation on which China's new history will be written, is relegated to a passing comment.

Often proffered as the ideal model for Third World development, China's growth has provided a burgeoning of material for commentators — so much so that it is often difficult to discern which of the opinions are really derived from Chinese experience and which are merely the theories of development experts. Thus, in a sentence

such as "Although the domination of science by politics during both these periods had an adverse effect on scientific development and on the economy in general, there was also a growing body of opinion that these massive campaigns were a necessary pre-requisite to generating political will for Mao-style development" (page 219), it becomes difficult to know which growing body of opinion is being considered.

Nevertheless, considerable printing errors apart, this volume of essays will remain a valuable source, as much a reflection of western social ideals in the 1970s, as of China's development. Not unexpectedly, for a collective work by different authors, written at different times and clearly aimed at different levels (a few no doubt originally intended as University lectures), there are repetitions of facts and discontinuities in style which detract from the overall impression of the book. But it does raise pertinent questions in the light of China's present changing policies: can similar development goals be achieved by roads other than that of revolution? The answer today may be very different from

that which seemed obvious not so very long ago.

The re-writing of any chapter of history, particularly when done at a distance, calls for no small amount of self-assessment. Leo Orleans, long respected as an analyst of Chinese affairs, expresses the new western dilemma: "It is possible to feel some regret that her [China's] drastic veering did not settle on some more middle course. Mao's dedication to the creation of an egalitarian society, his "serve the people" ethic, the sense of idealism he attempted to instill in the people, captured imaginations outside China as well as within, and some of those who disagreed with China's politics and policies could still identify with Mao's utopian ideals, [which] set China apart from every other nation" (page 225). By choosing a new and different road to development, China has set not only new goals for itself, but also a whole set of fresh criteria for western analysts. □

M. Christine King is researching into Chinese Affairs and the History and Philosophy of Science.

Higher marine fungi

R. A. Eaton

Marine Mycology: The Higher Fungi. By J. Kohlmeyer and E. Kohlmeyer. Pp. 690. (Academic: New York and London, 1979.) \$59.50; £34.60.

FOR 20 years, the authors' research in the field of marine mycology has produced a high quality of illustration and description of marine fungi, particularly through their *Icones Fungorum Maris* and *Synoptic Plates of Higher Marine Fungi*. A large part of this volume combines the essence of these publications to give us the most comprehensive work since Johnson and Sparrows (*Fungi in Oceans and Estuaries*, J. Cramer: Lehre, FRG, 1961) that any student of marine fungi would need.

More than half of the book comprises illustrated keys, and descriptions of higher marine fungi including 90 plates of micrographs. But is photographic representation the best means of illustration? Certainly the beautifully accurate line drawings in the *Icones* are not surpassed by many micrographs in this book. Their use of interference contrast microscopy introduces a new dimension for observing the appendaged ascospores of many marine fungi, but has its limitations in aiding interpretation of sectioned ascocarps or spermogonia, and non-appendaged spores.

Parasitic and saprobic marine fungi exist on various natural and man-made substrates from mangrove vegetation to polyurethane. They are distributed in different geographical zones and at different depths in the oceans. The book deals very comprehensively with the factors which affect the occurrence of filamentous fungi and yeasts in the sea, but the authors consider only a "selection of pertinent papers" on their physiology and biochemistry.

Other chapters are concerned with methodology, fruit body and spore ontogeny, and the release, dispersal and settlement of spores. The fascinating relationships which exist between fungi and marine animals (particularly marine wood borers) and between fungi and algae (producing marine lichens), are also covered. Apart from a chapter on marine yeasts this book is concerned wholly with filamentous higher fungi, as the title states. For the sake of completeness, was it not possible to broaden the scope of the book a little, and include more than a mention of the lower marine fungi — one-fifth of the total marine mycota?

The authors state that "a major objective of this treatise is to assist students of marine fungi in the identification of species." This is achieved, for it is unquestionably a reference book to be kept on the shelf above the microscope. □

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This book, dealing with one of the most exciting subjects in biology and medicine today, offers the first rigorous examination into the sites of "biological clocks" in animals, the mechanisms of action and their relationships to environmental factors.

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OBITUARY

O.R. Frisch, 1904-1979

ON 22 September 1979 Professor Otto Robert Frisch, OBE, FRS, Emeritus Jacksonian Professor in the University of Cambridge, died following an accident. He was a distinguished and original nuclear physicist. His contributions to physics will be, and the example he set in his approach to the subject should be, remembered by future physicists.

Both in physics and in music, which was next to physics his strongest interest, his main driving force was enjoyment of what he was doing. In physics this meant using simple apparatus, preferably designed and made by himself, and looking for important problems with transparent answers. Some of the instruments he made might be called gadgets, but surprisingly often these gadgets were able to provide answers to topical problems. Naturally "big physics" was not his line, and he never became involved with projects needing large machines and large teams of collaborators.

His piano playing was of near-professional standard, but he was not a perfectionist, and was not above listening to, or playing with, musicians of a much lower calibre, provided they shared his enjoyment of the result.

He was born on 1 October 1904 in Vienna. His father, Justinian Frisch, was a printer, his mother a very gifted musician. I do not know whether his father's profession influenced his interest in writing and his flair for the written word, but no doubt his mother encouraged his music. The members of the family were very close, and all his life he remained in touch with his relatives. Above all he was very close to his aunt, Lise Meitner, the famous nuclear physicist.

At school Otto Robert (as he was known to the family; later he was just Robert to his friends, except for the Los Alamos period, when there were too many Roberts around, and he became Otto) showed marked ability in mathematics, and almost chose it as a career, but in the end decided that this would be too abstract for his taste, and he settled for physics.

His studies in the University of Vienna under Professor Karl Przibram appear to have been uneventful, and he obtained his D Phil in 1926, after four years in the university. This was rapid progress, but at the time not unusual. After a year in a small firm run by an inventor, where he presumably could indulge in his love for gadgets, he accepted a post in the Physikalisch-Technische Reichsanstalt in Berlin. His work there was in the optics division, with the assignment to work on a proposed new light standard, but he says "I stayed long hours trying out half-baked



ideas of my own". His assignment was hardly exciting, but he was able to attend seminar meetings at the university, and listen to remarks by Einstein, Planck, Nernst and others. Lise Meitner was there, too. She worked, and lived, in the suburb of Dahlem, but Frisch saw her frequently, to the profit of his physics and his music.

In 1930 he became an assistant to Otto Stern in Hamburg, famous for the Stern-Gerlach experiment. Stern was an outstanding experimenter, not in manual skill, but in his command of the relevant principles and in the design of experiments, and his laboratory was the leading one in the world for atomic-beam research. Frisch worked closely with him and took part in important experiments, including the first observation of the diffraction of atomic beams by crystal surfaces. At the end of his Hamburg period he did his first important experiment on his own, observing the recoil of atoms on photon emission.

But by this time the Nazi government was in power, and both Stern and Frisch, who were of Jewish descent, had to leave. Frisch went to Birkbeck College to work under Blackett with a grant from the Academic Assistance Council. Here he became interested in nuclear physics, which in 1933 was full of excitement. He invented a method of measuring very short-lived activities, and this led to the discovery of some new radioactive isotopes.

A year later, Niels Bohr, who must have heard of his ingenuity in devising and carrying out experiments, invited him to Copenhagen. The next five years in the informal and stimulating atmosphere of

Bohr's Institute were very fruitful. It was the time when neutron physics had been initiated by Fermi's work, and Frisch, too, started on neutron work. Probably he was drawn in initially because he happened to have the most appropriate counter with which to test for neutron-induced activity. He made many important contributions to that subject.

It was in neutron physics that he did his most famous piece of work. He was spending Christmas 1938 with Lise Meitner, who was now in Sweden. She received a letter from Otto Hahn, with whom she had collaborated closely until she had to leave Germany, telling her of his discovery that barium was among the products emitted when uranium was bombarded with neutrons. Hahn realised this meant that the uranium nucleus must have split into two large pieces. Frisch and Lise Meitner were as dumbfounded by this news as all other physicists when they heard of it. But after thinking about the discovery for a while, they understood how the strong electric charge could help to overcome the surface tension and lead to an unstable configuration once the nucleus was set vibrating by a collision. If the nucleus had taken a sufficiently elongated form the mutual repulsion of the positive charges would drive it apart, and this would release an energy of about 200 MeV. Frisch coined the word "fission" for this process. After returning to Copenhagen and composing a letter to *Nature* jointly with Lise Meitner over the telephone to Stockholm, he carried out an impressively simple experiment proving the emission of energetic and heavily ionizing particles from the bombarded uranium, and thus confirmed the interpretation.

Fission was now the exciting part of nuclear physics, and most nuclear physics laboratories started to work on this. Frisch continued in this field, too, and did several more important experiments.

But this was 1939. War was imminent, and Denmark not too healthy a place for a German refugee. (The Anschluss had made him a German subject). Besides, the Danish police were making difficulties about extending his permit. So he tried to find a place in England, and, when Oliphant heard of his problem, he arranged an appointment for him in the University of Birmingham. Here Oliphant was trying to build up facilities for nuclear physics work, but there was not much equipment as yet, and Frisch could not continue his experimental work immediately. But he continued speculating about fission.

The possibility of the release of nuclear energy was then in many people's minds,

once it had been shown that neutrons were emitted after the fission. One was awed by the thought of a new weapon of terrifying power, but Frisch, like others who had understood the paper by Bohr and Wheeler on the mechanism of fission, and Bohr's argument that the fission caused by slow neutrons was due to the rare isotope ^{235}U , were reassured that no violent explosion could happen in ordinary uranium. The thought of separating uranium isotopes in large quantities seemed to belong to science fiction. Then one day Frisch came to me and said "Suppose one had a large amount of separated ^{235}U , what would happen?" One could make a guess at the fission cross section from the ideas of Bohr, and I knew how to estimate the critical size, given the cross section. The answer staggered us by being smaller than we would have guessed. Next we had to estimate how far the chain reaction would proceed before the energy it released would drive the uranium apart. The answer again staggered us by being a reasonable fraction of the total energy available. If that was so, we said, it would be, as a weapon, worth its price, even if the isotope separation plant cost as much as a battleship (this turned out to be an underestimate). What if the Germans got there first and a nuclear bomb was in the hands of Hitler's Germany?

We wrote down our arguments, and that was the start of serious interest in atomic energy work in England. Frisch first tried to explore the possibility of isotope separation by thermal diffusion, but did not get far, and we now know that the thermal diffusion coefficient in the only gaseous uranium compound happens to be practically zero. Instead Frisch moved to Liverpool, where in Chadwick's laboratory there was a cyclotron and other facilities for nuclear physics, and worked on the nuclear physics aspects of atomic energy until the end of 1943, when it was decided to discontinue work in England and move to America those who could be useful to the American work.

So Frisch went to Los Alamos, the strange atom-bomb city in the middle of New Mexico. He was not attached to any specialist team but had a roving assignment and in that way was able to give essential help to many different groups. One of the experiments he set up himself was a typically original scheme known as the dragon. Two pieces of fissile material which together would make a critical mass and so cause a violent chain reaction, were allowed near each other for only a short moment, by dropping one piece past the other. The speed was so arranged that the chain reaction did not develop to any dangerous degree. The name indicated that one was tickling the tail of a dragon. The experiment made it possible to observe details of a near-critical situation which were otherwise hard to get at.

After the war, Frisch returned to England and became head of the Nuclear Physics Division at Harwell. Adminis-

tration was not his favourite pastime, but he found that his deputy Dr (later Sir) Robert Cockburn delighted in running things and did it well. Frisch left Harwell in 1947 to become the Jacksonian Professor of Physics in Cambridge and a Fellow of Trinity. At that time nuclear physics in Cambridge was overshadowed by crystallography and other lines. It was not in Frisch's character to fight for more support. Besides, nuclear physics had now become big physics and less to his taste. While he kept a lively interest in nuclear physics and in the newly developing particle physics, he spent more of his time teaching and writing. Many students profited from contact with his way of doing and looking at things. He still liked gadgets, and made many ingenious instruments. His writing included *Meet the Atoms*, a very readable popular introduction to modern physics. Only a few months ago he published his recollections under the appealing title *What little I remember*,¹ and it is pleasing that he was able to leave this lively record of his personality.

Shortly after moving to Cambridge he married, and there were two children. His wife shared not only his Austrian origin, but very many of his attitudes and his love for music.

Shortly before retiring in 1972 he invented an apparatus for measuring and evaluating bubble-chamber tracks, and he later became a partner in a firm set up to manufacture this. I do not know whether this commercial activity made him a wealthy man, but he clearly enjoyed this novel position and seeing his gadget being produced.

His was a full life, but he just failed to reach his seventy-fifth birthday with its warm messages from his many friends.

Rudolf Peierls

1. Frisch, O. R. *What Little I Remember*. (Cambridge University Press, 1979) See review by Rudolf Peierls in *Nature* 280, 257-259 (19 July 1979).

Martin Lüscher

IN THE midst of his fruitful work, Martin Lüscher's life was taken on 9 August 1979 by a tragic accident, at the age of 63. He was Professor of Zoology at the University of Berne, and directed its Department of Zoophysiology with scientific foresight and a fatherly concern for his colleagues and students. His name will be remembered by zoologists throughout the world.

Martin Lüscher, son of the Basle artist Jean-Jacques Lüscher, spent a quiet youth in his family home, with extended visits to Provence. There, the harmony and beauty of the landscape created a lasting impression on him; in the balmy

Mediterranean atmosphere with the colour play and diverse insect cacophony in the flower fields he developed a love and interest of plants and animals. In Basel in 1944 he was awarded a PhD in zoology. After his marriage to Noemi Stoecklin, daughter of the Basle artist Niklaus Stoecklin, which proved to be a true life partnership, he began, with her, his life-long progress as a zoologist. They shared a life of work, pleasures and trials. A great deal of his professional success was due to his close accord with his wife, with guests always welcome at their home.

As a young zoologist, Lüscher's first post was as a research assistant in Berne, where he worked under F.E. Lehmann on the physiology of development of amphibians. Soon afterwards he met his great teacher, the insect physiologist, Sir Vincent Wigglesworth of Cambridge, under whom he was able to work. Martin Lüscher saw Wigglesworth as the founder of a new branch of research: experimental insect physiology.

From England Lüscher moved to Paris, where he was introduced to the biology of termites by the renowned termite specialist T.P. Grassé and found the stimulus for what was to become his principal research — caste formation in termite colonies. He wished to study termites in the field as well as the laboratory, and he soon had the opportunity to take part in an expedition to observe at first hand the highly organised termite colonies of Africa. Lüscher was particularly fascinated by the highly elaborate nest structures built by the millions of termites within the colony. He was the first to understand their design as a well planned respiratory system with temperature and humidity regulation, and an air circulation system functioning through the warming of the air inside the nest. After imaginative and productive field work he spent a very important year of study in the United States on a grant from the Rockefeller Foundation. He was given the opportunity to stay for some time with A.E. Emerson, the expert on termite biology and taxonomy, and was then able to acquaint himself with modern methods of insect endocrinology in the leading laboratories at Berkeley, and at Harvard with C.M. Williams. Afterwards he worked at the Swiss Tropical Institute in Basel and in 1954 was appointed professor at the University of Berne. Under his direction the new Department of Zoophysiology was formed.

In 1965 he was appointed to the four-year directorship of the Zoological Institute. During the academic year 1967/68 he was Dean of the natural science faculty and from 1969 a member of the Swiss National Science Foundation. In his last important undertaking, in Nairobi as project leader at the newly founded International Centre of Insect Physiology and Ecology, he set up an enthusiastic research team.

Lüscher carried his title with quiet

modesty. His lectures were simple and clear, and his criticism was always constructive and helpful. His motivation as a researcher derived from the pleasure of observation, an urge for knowledge and a keen understanding of relationships. He was intrigued, like many earlier researchers, by the secretive world of the termite colony. How do all the different castes including the workers, the soldiers and the reproductive individuals develop from the same origin in such numbers? How is this ratio maintained and regulated? His understanding of the termite colony as one organism, which he had gained in Africa, and the methods of experimental physiology he had learned, led to further discoveries. He found that all the castes of the termite colony interact continuously, as though they were parts of a single organism. The presence of reproductives in lower termite colonies inhibits the development of that caste, whereas their absence induces the formation of further reproductives. In a similar way the ratio of developing soldiers is regulated by the proportion of soldiers present in the colony.

Lüscher became aware of the importance of 'social hormones' as agents of regulation and communication within the termite colony, and with the biochemist Peter Karlson he introduced the now well-known term pheromone. He could demonstrate by means of ingeniously simple experiments that pheromones are released from the anus of the reproductive individual and are ingested orally by other termites and then distributed throughout the colony by oral exchange, the so-called process of trophallaxis. Pheromones regulate the make up of the social structure, whereas hormones determine the process and direction of development of the single individual, and the latter are controlled by the former. It was established experimentally that the inhibition of reproductive caste development could be simulated by application of 'juvenile hormone', which in general inhibits the metamorphosis of larval insects into adults. The same hormone applied in higher doses to the termite society was shown to stimulate soldier production. With these findings, Lüscher made a breakthrough towards a new physiological understanding of termite caste regulation. The antagonistic effect of reproductives and soldiers that inhibit the development of their own caste and open the way towards development of the other could be interpreted physiologically: the reproductive castes transmit, by means of their pheromones, information causing the developing larvae to produce their own juvenile hormone, whereas the soldiers, probably also by means of specific pheromones, transmit information to inhibit juvenile hormone.

Recent results from Lüscher's team in Nairobi and Bern suggest similar exocrine-endocrine principles of caste regulation for

the highly organized mound-building 'higher termites.'

To understand all these interrelationships Lüscher augmented his work with basic research into the mechanism of hormonal interaction. He investigated the mechanisms of action of juvenile hormone and the ecdysteroid moulting hormones. He studied the interactions and regulatory mechanisms of those hormones in the cockroach, a nonsocial insect related to termites.

Lüscher's name as a termite researcher and insect physiologist has international acclaim. He retained, however, his love for the countryside, for plant and animal life and for his fellow men. I best remember the hours I spent with him in the African bush, as we stood looking with astonishment at an opened termite mound. Here lay the motivation and drive for his life's work: the questions arising from his awe and respect at the incomprehensible and beautiful. This ethos together with scientific accuracy and genius gave him and his work its far reaching importance.

Reinhard Leuthold

G.S. Adair

GILBERT SMITHSON Adair, who died on 20 June 1979, was a highly respected and well loved character on the Cambridge scene for sixty years. Many generations of students and research workers have cause to be grateful for his willing help with practical advice and theoretical explanations. This ranged from supplying Max Perutz with his first haemoglobin crystals and instructing people in the art of preparing collodion membranes of varying permeabilities, to simple explanations of Gibbsian thermodynamics. In this way he exerted considerable influence although he was, to the best of my knowledge, only formally responsible for the supervision of two research students; Muriel Robinson (who became Muriel Adair) and the author of this notice.

Adair was born on 21 September 1896, the son of a Quaker family. He went up to King's, Cambridge, in 1915 and remained in that city, with only very brief interruptions until his death. He was a Fellow of his college from 1923 to 1933 and an honorary Fellow after retirement from his University post (Reader in Biophysics) in 1963.

The wide interest of biophysicists in haemoglobin, which extended over the last 60 years, involved several distinguished Cambridge men. Adair had the stimulation of A.V. Hill, Barcroft and Roughton, who not only contributed to our knowledge about the respiratory function of haemoglobin, but also used this important

and plentiful protein to develop novel thermodynamic and kinetic methods. The iron contents had given an equivalent weight for haemoglobin of about 16,700 Daltons. Brown and Hill had shown that comparison of calorimetric and van't Hoff ΔH for oxygen binding gave about 17,000g of haemoglobin per mol of binding site. Osmotic pressure measurements preceding those of Adair gave variable results and tended to support the prevailing theory that haemoglobin occurred in solution with a molecular weight of about 17,000 and that there was some tendency towards aggregating of these units. Adair's attention to experimental detail is illustrated in a masterly paper "A critical study of the direct method of measuring the osmotic pressure of haemoglobin" (*Proc. Roy. Soc. A108*, 627, 1924) in which he reports a molecular weight of 67,000 and explains the erroneous results of others. It is important to point out that this preceded by about a year, Svedberg's determination of the molecular weight of haemoglobin in the ultracentrifuge.

Further studies of the osmotic pressure of haemoglobin solutions led Adair to detailed experimental and theoretical investigations of membrane potentials. In the 1930s he extended these studies to serum proteins and to the determination of charges on protein molecules from the measured membrane potential at different pH and ionic strength.

The above contributions led to his election to the Royal Society in 1939. During the war Adair was much occupied with the teaching of physiology, especially in practical classes. After the war, one of his main concerns was to improve his simple technique for measuring osmotic pressures, so that small pressures of solutions of large protein molecules could be measured. He also found it difficult to believe the results of his second research student, which indicated reversible dissociation of haemoglobin molecules in dilute solutions and at high ionic strength, until he confirmed it himself with his refined methods.

Adair was a shy but very friendly man who was absolutely devoted to his rather specialised, but very valuable work in the laboratory. The essence of his experimental technique was simplicity and attention to detail. Old fashioned alarm clocks and domestic heaters formed the backbone of his wonderful method for making semi-permeable membranes.

Apart from his scientific work Adair read widely and, in his youth, was a keen rock climber. There are a number of stories about his climbing activities at college and into the Physiology Laboratory. From 1931 until his death in 1977, he shared a life of work and simple pleasures with Muriel Adair. Their house near Grantchester Meadows allowed him to pursue his interests in the observation of a variety of plant and animal life.

H. Gutfreund